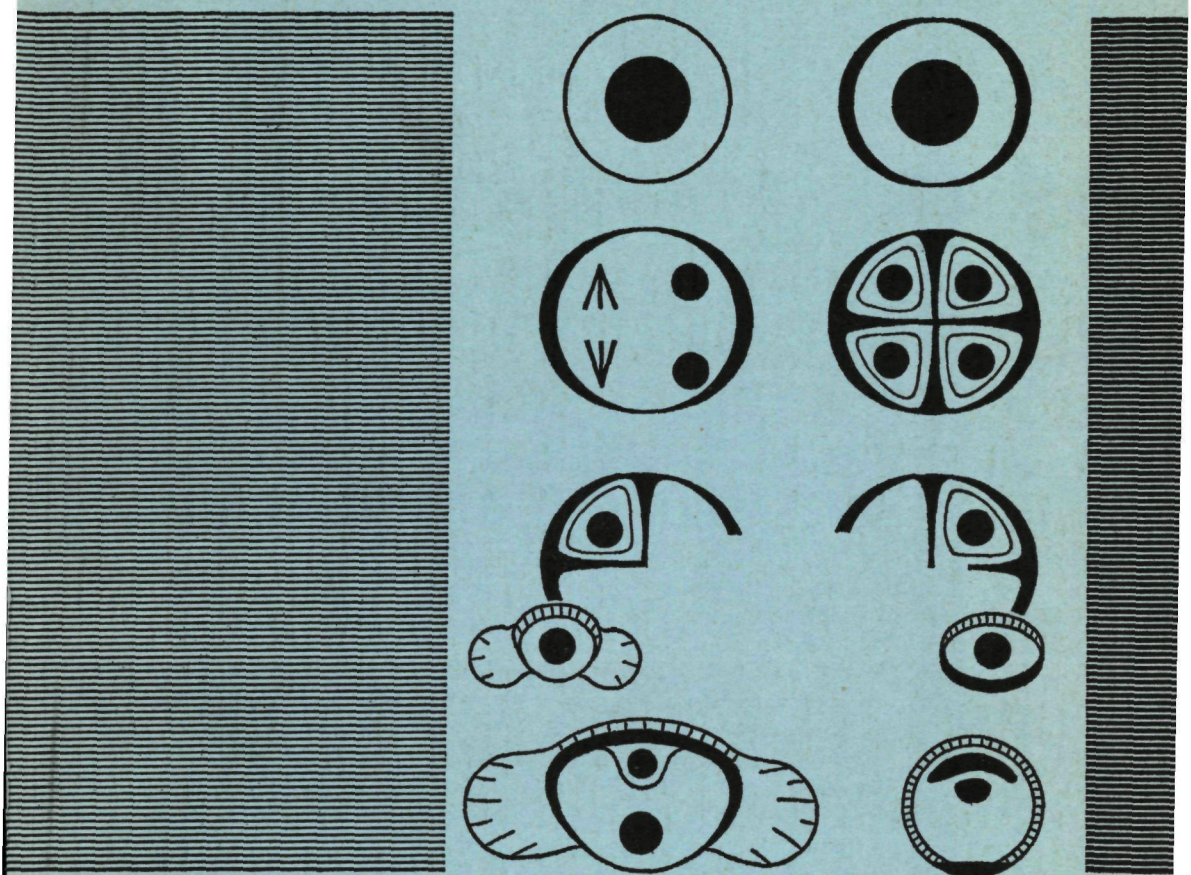
M. Th. M. Willemse



MICROSPOROGENESIS IN PINUS SYLVESTRIS AND GASTERIA VERRUCOSA

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A comparative morphological study and a quantitative approach on submicroscopical level

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A comparative morphological study and a quantitative approach on submicroscopical level

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
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Ter nagedachtenis aan mijn vader
Aan mijn moeder
Aan Annemie en Nicole
Aan Oom Niek

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INLEIDING

Microsporogenese is het ontstaan van een cel, die in staat is haploid weefsel te vormen, waaruit mannelijke geslachtscellen ontstaan. Dit weefsel is bij hogere planten beperkt tot enkele cellen Door deze sterke reductie van het haploide weefsel is de vorming van de mannelijke geslachtscel nauw verbonden met de microsporogenese Bij lagere plantensoorten, zoals bruinwieren of mossen, ontstaat uit de microspore vaak een zelfstandig organisme met speciale organen, die de geslachtscellen produceren In een studie over microsporogenese bij hogere planten is de vraagstelling vooral gericht op de vele specialisaties, die de microspore ondergaat met betrekking tot de vorming van de mannelijke geslachtscel en het voortplantingsproces

Microsporogenese bij Spermatophyta, dat wil zeggen de vorming van pollenkorrels bij zaadplanten, is voornamelijk beperkt tot enkele facetten voor enkele organismen beschreven In de veelal morfologisch gerichte studies gaat de interesse bij voorkeur uit naar de vorming van de speciale wand van de pollenkorrel De soortseigen sculptuur en de bijzondere chemische samenstelling van de pollenwand zijn belangrijke eigenschappen, die hun toepassing vinden in de palynologie en fylogenetisch gerichte studies Als object van onderzoek van de microsporogenese prefereert men vanwege hun grootte, vertegenwoordigers van het geslacht *Lilium* (Heslop-Harrison 1968, Dickinson 1970) of *Tradescantia* (Maruyama 1968, Mepham & Lane 1970) Sinds kort zijn enkele gymnospermen onderzocht, met name *Podocarpus macrophyllus* (Vasil & Aldrich 1970) en *Pinus banksiana* (Dickinson & Bell 1970) In aansluiting hierop is voor de hier gepresenteerde studie een vertegenwoordiger van de gymnospermen, *Pinus sylvestris*, en een lelieachtige, *Gasteria verrucosa*, gekozen Bovendien zijn deze organismen, zij het een beperkte tijd, ruimschoots voorhanden.

De microsporogenese is bij *Pinus sylvestris* uitvoerig onderzocht vanaf het zygoteen tot en met het jonge microspore stadium en bij *Gasteria verrucosa* vanaf de pollenmoedercel tot en met de rijpe pollenkorrel De zich ontwikkelende microsporen en de daaromheen gelegen tapetumcellen zijn bestudeerd op submicroscopisch niveau, waarbij aandacht is besteed aan de veranderingen, die zich voordoen in de celkern en het cytoplasma Getracht is de optredende veranderingen te correleren aan de celprocessen, die tijdens de verschillende fasen van ontwikkeling plaatsvinden Opvallend zijn de vorming van de speciale uit callose gevormde celwand rondom de microsporen en de aanleg en opbouw van de pollenwand, die voornamelijk bestaat uit sporopollenine Tijdens de ontwikkeling en onder invloed van verschillende chemische behandelingen zijn de samenstelling en veranderingen in de pollenwand bestudeerd door een analyse van de zelffluorescentie van de pollenwand, die optreedt bij bestraling met ultraviolet licht

In een kwantitatieve benadering van de microsporogenese zijn de veranderingen van het celvolume en de dichtheid van plastiden, mitochondriën, vetdrup-

peltjes en Golgi-lichaampjes per eenheid van cytoplasma-oppervlakte beschreven

Van *Pinus* en *Gasteria* zijn de verkregen resultaten met reeds bekende gegevens vergeleken De overeenkomsten en de verschillen in het proces van de microsporogenese van beide, in fylogenetische zin uiteenliggende, planten zijn geïnterpreteerd

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MORPHOLOGICAL AND QUANTITATIVE CHANGES IN THE POPULATION OF CELL ORGANELLES DURING MICROSPOROGENESIS OF PINUS SYLVESTRIS L. I. MORPHOLOGICAL CHANGES FROM ZYGOTENE UNTIL PROMETAPHASE I.

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SUMMARY

The membrane-like structures in the nucleus, the nuclear pore, the disappearance of the nuclear membrane, the synaptinemal complex, and changes in the nucleolus and karyoplasm are described and discussed.

Plastids and mitochondria do not change during the microsporogenesis. Until diplotene the lipid granules with electron transparent vesicles are arranged into a "lipid complex". During pachytene rough endoplasmic reticulum changes into smooth endoplasmic reticulum. During diplotene numerous Golgi bodies are present. Microtubules are present from zygotene and scattered in the cytoplasm. At diakinesis many vesicles appear. Ribosomes as well as polysomes are present. From diakinesis only ribosomes are observed.

Callose wall formation starts in diplotene and is connected with the presence of many smooth endoplasmic reticulum cisternae and Golgi vesicles, probably also with polysomes and lipid granules.

1. INTRODUCTION

The most striking process during microsporogenesis is the formation of the callose wall and the pollen wall. Numerous submicroscopical investigations have been made on pollen of various plant species in the successive stages from meiosis to mature pollen.

In Monocotyledons the structure of the synaptinemal complex in the nucleus during the meiotic prophase does not differ much from that in animal organisms (SOTELO 1969), as appeared from investigations with *Lilium*, variety Cinnabar (ROTH & ITO 1967) and *Allium cepa* (STOCKERT *et al.*, 1970). The changes of the nucleolus during cell divisions have been described for different organisms (MILLER & BEATTY 1969). A loss of nucleolar material during cell division has been supposed by BAJER & MOLÈ-BAJER (1969) in *Haemanthus katherinae*, and by DICKINSON & HESLOP-HARRISON (1970) in *Lilium henryi*. In pine, the structure of the nuclear pore is very complex. Morphologically it is different from the pore in amphibian oocytes as described by WISCHNITZER (1958) and FRANKE & SCHEER (1970). As is shown by different descriptions of the nuclear pore, its structure is not fully known (ABELSON & SMITH 1970). During cell division the nuclear membrane is partly broken down and its fragments remain in the cytoplasm (ESAU & GILL 1969).

The morphological changes of the amyloplasts, mitochondria and the Golgi body in the cytoplasm during microsporogenesis have been studied in *Tradescantia paludosa* (MARUYAMA 1968). Changes occur in the starch content of the amyloplasts, mitochondria divide during pachytene.

Before the breakdown of the nuclear membrane the microtubules are oriented in the cytoplasm in a prophase band (NEWCOMB 1968) or in distinct regions, as has been found in *Dactylorchis fuchsii* (BURGESS 1970).

The formation of the callose wall has been described for *Cucurbita ficifolia* by ESCHRICH (1964) and for *Endymion non-scriptus* by ANGOLD (1967). ANGOLD supports the opinion that some relationship should exist between the smooth endoplasmic reticulum (SER) and the callose production.

The present study deals with the morphological changes during microsporogenesis in *Pinus sylvestris* from the zygotene until the prometaphase I. As many events and morphological structures are similar to those in other plant species, comparisons can easily be made.

2. MATERIAL AND METHODS

During the first half of May microsporophylls of the cone of *Pinus sylvestris*, originating from one tree, were collected. Small pieces were fixed for one hour in 1% OsO_4 at 0°C in phosphate buffer pH 7.2. During the time of fixation the specimens were shaken continuously. After 15 minutes washing in water the specimens were stained for 30 minutes in 1% uranyl acetate in water, followed by another 15 minutes washing in water, and were put during 30 minutes into 1% $KMnO_4$ in water. After dehydration in alcohol the specimens were embedded in Epon 812. Sections of 80 nm thickness were cut using a Porter-Blumm ultramicrotome. After 5 minutes staining with lead citrate (REYNOLDS 1963), the material was examined with the Philips EM 300 electron microscope at 60 KV. The different stages were determined by observing 1 μm sections under the phasecontrast microscope.

3. RESULTS

Just before meiosis starts, the pollen mother cells produce a new thin cell wall inside the normal cellulose wall. This new cell wall consists of two electron dense layers of thin fibrillar material with fibrils between these layers (*fig. 19, 26*). The cellulose wall disappears when meiosis begins, whereas the new thin wall remains around the cell.

3.1. Zygotene

The large nucleus has homogeneous nucleoli and the chromatin shows signs of contracting. During late zygotene irregularly shaped structures are present between some of the contracting chromatin masses which structures are visible only for a short time (*fig. 1*). The thickness of the surrounding "membrane" is approximately 11 nm. Sometimes the "membrane" has blebs. Inside the circular

structure there is an electron transparent thin fibrillar material, possibly chromatin fibrils, which makes contact with the membrane-like boundary (*fig 3*) It seems that the "membranes" become visible only after the formation of the electron transparent space between the contracting chromatine masses (*fig 2*)

The nuclear pore is very complex and has the same structure in all stages of microsporogenesis In the electron dense annulus about eight circular regions are present with a diameter of approximately 18 nm The centre of the pore contains electron dense material (*fig 4*) In cross section small fragments of the outer nuclear membrane extend to both sides above the pore (*fig 5*) They do not occupy the whole outline of the annulus, but are situated between the eight regions, thus every pore on both sides has eight extensions These extensions are tubular with a diameter of 7 nm The annulus of the pore is composed of eight very small tubules on both sides in a regular arrangement The tubules have probably closed ends In the nucleus thin fibrils are connected with the pore (*fig 7*) *Figure 9* presents a drawing of the supposed structure of the nuclear pore

During zygotene and pachytene the synaptinemal complex is visible in a number of places (*fig 8*) Two lateral arms, the intermediate space and the medial ribbon are clearly distinguishable, especially during late zygotene (*fig 10*). In pachytene the lateral arm is not easily discernible and is probably continuous with the granular chromatin Bridges between the lateral arm and the medial ribbon become less numerous compared with the situation during late zygotene (*fig 11*) Finally, during diplotene only an electron transparent oblong band with some electron dense material is observed between the chromatin which disappears rapidly

The plastids in the cytoplasm have a granular content and rarely contain other elements like little fragments of membranes Some plastids have a big starch granule There are also plastids which have a thin long centre piece, which is characteristic for dividing plastids The mitochondria contain some osmiophilic material and have few cristae Numerous Golgi bodies are present, but they possess few vesicles only (*fig 12*)

The lipid granules have osmiophilic dots on their edges, which become visible after poststaining with lead The lipid granules are connected with large electron transparent vesicles and the whole group is also surrounded by dark dots This "lipid complex" is visible until diplotene and appears again in the tetrad stage (*fig 13*)

Rough endoplasmic reticulum (RER) membranes are assembled in packets, and in some places they are not completely covered by ribosomes These packets of RER are characteristic during zygotene and pachytene (*fig 16*) Microtubules are rare and are situated mainly against the nuclear membrane Large numbers of ribosomes and some polysomes give a grey tint to the cytoplasm (*fig 7, 13, 16*)

3 2 Pachytene

The karyoplasm is highly electron transparent The nucleolus is still homogeneous (*fig 8*) In the cytoplasm the dark dots around the lipid granules disappear

and the "lipid complex" begins to disperse. In this stage the Golgi bodies possess more vesicles, but they are still small in size (*fig. 14*). Packets of RER are observed repeatedly, the lumen of the membranes shows dilatations and the ribosomes on the membranes are lacking in some places. More SER is present (*fig. 15, 17*). In all directions and everywhere in the cytoplasm microtubules become visible. Ribosomes as well as polysomes are present (*fig. 15*).

3.3. Diplotene

During early diplotene the karyoplasm contains very thin fibrillar material and electron dense granules. Locally it shows light zones (*fig. 18, 23*). After the initial phase the granules between the contracting chromosomes increase in number (*fig. 19, 28*). The nucleolus, which was initially homogeneous (*fig. 18*), becomes more heterogeneous and is connected with less electron dense granular material (*fig. 20, 21*). The more electron dense part of the nucleolus has the same structure as the electron dense bodies, which become perceptible in the cytoplasm from diplotene until interphase I (*fig. 20, 22, 26*). This part has often granules on its margin (*fig. 21*). Finally the nucleolus shifts towards a reticulate form (nucleolemma) and disappears (*fig. 19*).

In comparison with the preceding phases the plastids and mitochondria do not change. The cytoplasm has electron dense lipid granules with dark dots and less electron dense lipid granules without dark dots. The Golgi bodies are present in large numbers and produce many small and some large vesicles (*fig. 24, 29*). SER is dispersed in the cytoplasm, RER strands are scanty. The microtubules are stretched in all directions. Polysomes as well as ribosomes are present (*fig. 25, 26*).

During diplotene the callose wall formation starts. The small space between plasma membrane and cell wall contains only some small granules and fibrils (*fig. 27*). The small space grows when the callose wall formation begins. Outside the plasma membrane showing sometimes undulations, a fine electron dense fibrillar material has accumulated against the cell wall (*fig. 26*). Thereafter a fine fibrillar network against the cell wall becomes visible, which changes into a highly electron transparent line between the cell wall and the flat plasma membrane (*fig. 28, 29*). The callose wall envelops the whole cell and grows in thickness until the tetrad stage (*fig. 30*).

At the start and during the callose wall formation many SER cisternae and Golgi vesicles are present in the cytoplasm (*fig. 24*). Between the plasma membrane and cell wall the material of the growing callose wall has a similar structure as the content of the cisternae of the SER and Golgi vesicles (*fig. 24, 26, 29*).

3.4. Diakinesis

In early diakinesis the nuclear membrane shows great undulations. The chromosomes are visible as blocks of electron dense granular material. The karyoplasm possesses granules with a size of about 30 nm and less numerous of 15 nm (*fig. 32*). The breakdown of the nuclear membrane starts with a local widening

of the perinuclear space. Thereafter, the membrane structure fades; here very small pieces of membrane remain in an osmiophilic zone (*fig. 31*).

No morphological change occurs in the plastids and mitochondria. The Golgi bodies produce small vesicles. Lipid granules are less electron dense and lack their surrounding dark dots. Vesicles with an electon transparent content are numerous. Round and large vesicles with a clear membrane are present during all phases. During diakinesis and the following metaphase, however, there are also very dilated cisternae of SER and many small vesicles (*fig. 34*). Some microtubuli are situated perpendicular on both sides of the disappearing nuclear membrane (*fig. 31*). In the cytoplasm and karyoplasm more microtubules are oriented parallel; in cross section some microtubuli are surrounded by an electron transparent core (*fig. 32, 33*). Ribosomes are distributed in the cytoplasm, polysomes are not observed in this stage.

4. DISCUSSION AND CONCLUSION

4.1. The nucleus

In the karyoplasm membrane-like structures between the contracting chromatin may be caused by a change of molecular charge due to the contraction of the chromatin. Thereafter a demixture and separation between karyoplasm and chromatin takes place. In the region of this demixture there could be a reorientation of molecules according to their charge. For a moment thin films could be formed locally which are visible as membrane-like structures. The whole process may be analogous to the formation of coacervate droplets (BUNGENBERG DE JONG 1949).

During pachytene the karyoplasm is highly electron transparent, granules of approximately 30 nm appear in diplotene. Such granules, but also granules of 15 nm, increase in number during diakinesis. Mixing of these granules with the cytoplasm takes place after telophase I. When the granules in the karyoplasm appear, the nucleolus shows some changes. The heterogeneous nucleolus has granules on its margin. MILLER & BEATTY (1969) found granules around the nucleolus in oocytes of *Rana calamitans*, which were apparently related to the nucleolar RNA metabolism. These granules may be considered as the 32S RNA particles which are produced in the nucleolus and become visible later as larger granules in the karyoplasm. It may be possible that in pine the granules in the karyoplasm have originated from the nucleolus. The more electron dense part of the nucleolus in diakinesis has the same structure as the electron dense bodies in the cytoplasm during this phase. Dense bodies derived from the nucleolus are also found in the cytoplasm of the dividing endosperm cell of *Haemanthus* (BAJER & MOLÈ-BAJER 1969) and during meiosis in *Lilium* (DICKINSON & HESLOP-HARRISON 1970). The changes in the karyoplasm during meiotic prophase are also related to the contraction of the chromatin and nucleolar activities during diplotene.

In pine the ultrastructure of the synaptinemal complex is in agreement with numerous descriptions in animals and plants (ROTH & ITO 1967; SOTELO 1969;

STOCKERT, GIMENEZ-MARTIN & SOGO 1970) Remarkable is the high number of synaptinemal complexes during zygotene and pachytene The medial ribbon is composed of two layers as is found in *Periplaneta americana* (SOTELO 1969)

The annulus of the nuclear pore is an arrangement of eight small circular regions which are situated between eight very small tubular extensions on the outer nuclear membrane These tubules are bowed and extend on both sides of the pore An octagonal pattern of the nuclear pore has been reported by WISCHNITZER (1958) in amphibian oocytes The author supposes that the annulus possesses eight microcylinders An octagonal pore in amphibian oocytes has been described by GALL (1967) and in *Haemanthus* by BAJER & MOLF-BAJER (1969) FRANKE & SCHEER (1970) suppose that the eight subunits in the annulus are granules, on the contrary, ABELSON & SMITH (1970) are of the opinion that the subunits are minitubules

In pine the eight small circular structures are the regions between the eight thin tubules It cannot be excluded that in pine another type of a nuclear pore is present, the dimensions of the pore are smaller than in amphibian oocytes (GALL 1967) The diameter of the thin tubules, about 7 nm, fits the thickness of the unit membrane

Before breakdown the nuclear membrane shows undulations Dilatations between the two membranes become visible and locally the two membranes become diffuse Parts of the nuclear membrane remain intact At places where the breakdown, probably by enzymes, takes place, sometimes small membrane fragments are visible Thus a local breakdown exists (ESAU & GILL 1969)

4 2 The cytoplasm

Until prometaphase I the plastids and mitochondria in pine do not change in their morphology The plastids have an electron dense content or they may contain a large starch granule On the contrary, in *Tradescantia* the size of the starch granule in the plastids increases after the leptotene up to the young microspore (MARUYAMA 1968) In the mitochondria few cristae are present Dividing plastids as well as mitochondria are found in all stages

The "lipid complex" decomposes after zygotene The black dots around the lipid granules are visible after poststaining with lead and sometimes are spread out over other organelles lying in the vicinity of the lipid granule Therefore these dots are probably artifacts, closely connected with the lipid granules

Vesicles in the cytoplasm have a clear electron dense membrane and a rather large size The high number of vesicles just before the division is due to the presence of SER cisternae and small Golgi vesicles, which ar more dilated now

The Golgi bodies produce vesicles They increase in number during pachytene and diplotene, and this augmentation may possibly be related to the formation of the callose wall

The RER is mainly perceptible in groups of membranes After zygotene the RER has lost its ribosomes and in diplotene only SER is visible

During zygotene until diakinesis the microtubules are distributed at random in the cytoplasm Contrary to what has been found in mitotic cells (NEWCOMB

1969), in pine no prophase band exists, and the microtubules are not oriented in distinct regions (BURGESS 1970). The number of microtubules increases until prometaphase. During the breakdown of the nuclear membrane the microtubules penetrate the karyoplasm at right angles to the disappearing nuclear membrane. Possibly this penetration of microtubules into the nucleus occurs as a result of a destruction and rebuilding of the microtubuli (ESAU & GILL 1969).

In all stages ribosomes are visible, contrary to the situation in *Lilium* (MACKENZIE & HESLOP-HARRISON 1967) where the ribosomes disappear during pachytene. In *Petunia* (LINSKENS 1969) and *Trillium* (HOTTA & STERN 1963) a decrease of the RNA content in pachytene is demonstrated as well. It appears from histochemical studies in *Cosmos* by KNOX, DICKINSON & HESLOP-HARRISON (1970), that RNA disappears between zygotene and pachytene; this does not necessarily imply that also the ribosomes should be absent in the cytoplasm. Up to diakinesis in pine polysomes are present.

4.3. The callose wall formation

A small zone of thin electron dense fibrils, which converts into an electron transparent line, is the first sign of callose wall formation during diplotene. The plasma membrane sometimes has local undulations and vesicles are found near this membrane. But the plasma membrane is principally flat and lies near to or in direct contact with the coming callose wall. The plasma membrane in pine is always intact, contrary to that in *Cucurbita* (ESCHRICH 1964).

Many cisternae of SER are present in the cytoplasm and possibly a relation exists between these cisternae and the formation of the callose wall, as ANGOLD (1967) has reported for *Endymion*. In pine the SER is possibly derived from the RER. The presence of Golgi vesicles lying near the plasma membrane may be connected with the callose wall formation and their content may be excreted. A high number of polysomes in the cytoplasm is observed and a decomposition of the lipid complex occurs during callose wall formation. It is remarkable that all these phenomena are repeated during the callose wall formation in the tetrad. For this reason the callose wall formation will be fully discussed with the description of that phase.

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LEGENDS TO THE FIGURES

Fig 1 Zygotene irregularly shaped structures among the chromatine (Ch), × 7,900
Fig 2 "Membranes" (arrows) appear after demixing, × 31,000
Fig 3 "Membranes' with blebs, the structure contains fibrils, × 22,000
Fig 4 Annulus of the nuclear pore with circular regions (arrows), × 103,000
Fig 5 Extensions on both sides of the outer nuclear membrane (arrows), × 103,000
Fig 6 Regular octagonal arrangement of the tubular extensions, × 207,000
Fig 7 Nuclear pores with annulus, thin fibrils in the nucleus are connected with the pore (arrows), near the nucleus microtubules (m) and ribosomes (r), × 27,600
Fig 8 Pachytene nucleus with synaptinemal complexes (Sc) and homogeneous nucleolus (n), × 5,000
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Fig 11 Synaptinemal complex during pachytene, × 24,100
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Fig 15 Pachytene cytoplasm with ribosomes (r), SER (S) and microtubules (m), × 47,200
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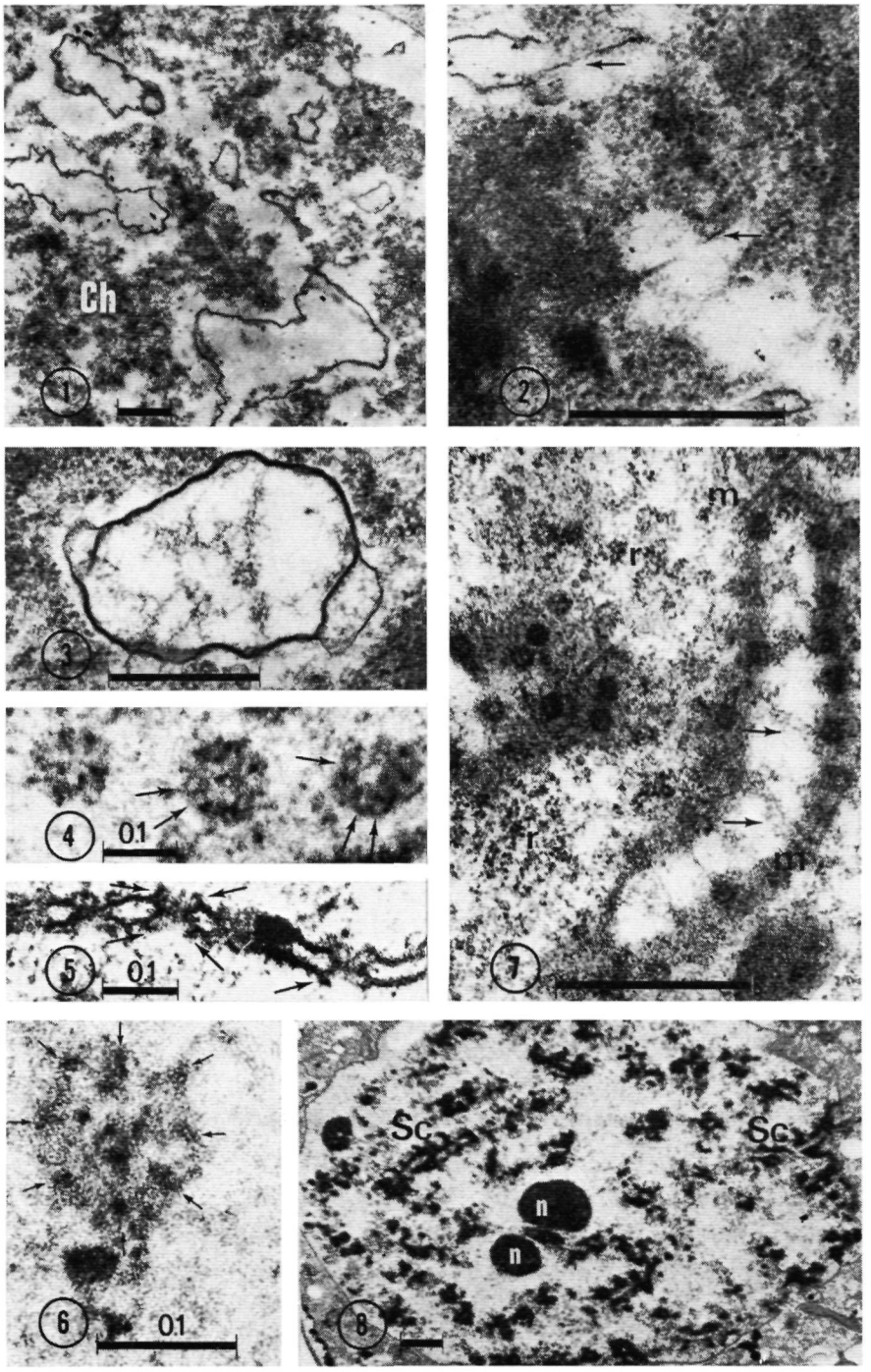
Fig 19 Diplotene karyoplasm becomes granular (g), the nucleolus (n) is disappearing, × 5,100
Fig 20 Heterogeneous nucleolus with fine granular material (arrow), × 14,600
Fig 21 Nucleolus with fine granular material (arrow) and granules (g), × 16,800
Fig 22 Dense body in the cytoplasm, × 25,200
Fig 23 Early diplotene karyoplasm with granules (arrow), × 23,000
Fig 24 Diplotene cytoplasm with Golgi bodies (G), Golgi vesicles (v) and SER (S), × 32,800
Fig 25 Distribution of microtubules (m), × 28,350
Fig 26 Callose wall formation starts with the appearance of fibrillar material (arrow) between the plasma membrane (pm) and the thin cell wall (C) The cytoplasm contains a dense body (db), ribosomes (r) and polysomes (p), note the content of the vesicle near the plasma membrane (arrow), × 31,600
Fig 27 The space between plasma membrane and cell wall before callose wall formation starts, × 21,900
Fig 28 Thin fibrillar material of the growing callose wall (arrow), × 27,600
Fig 29 A thin electron transparent line appears Note the content of the Golgi vesicles (arrows), 47,400
Fig 30 The callose wall (Ca) during prometaphase I, × 32,200
Fig 31 Diakinesis breakdown of the nuclear membrane, small pieces remain in an osmiophilic zone (arrows), microtubules (m) at right angles to the disappearing membrane, × 55,200
Fig 32 Diakinesis karyoplasm with granules (arrows), × 36,800
Fig 33 Parallel oriented microtubules (arrows), × 36,800
Fig 34 Large vesicles (V), small (v) and irregularly shaped vesicles (arrow) during diakinesis, × 15,750

Unless mentioned otherwise, the line on the figures represents a length of 1 μm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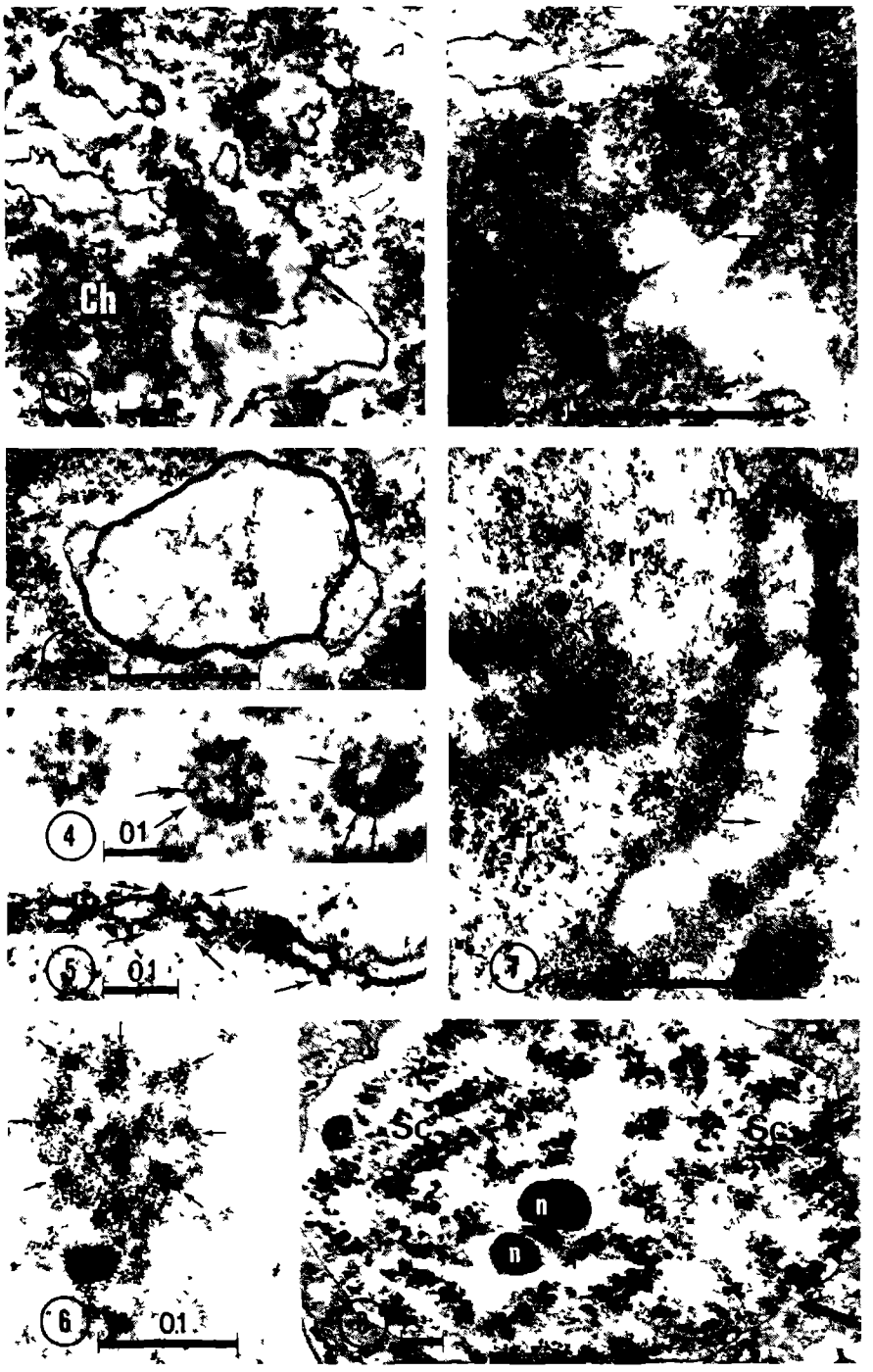
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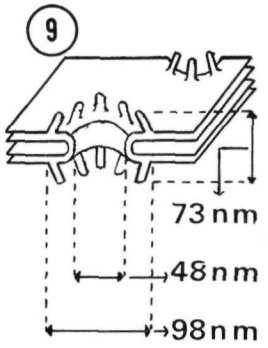
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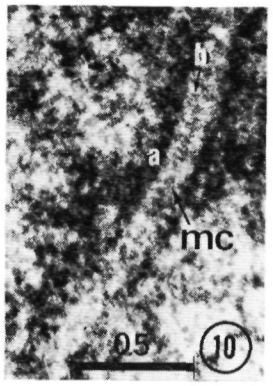
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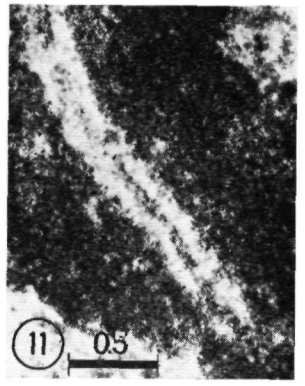
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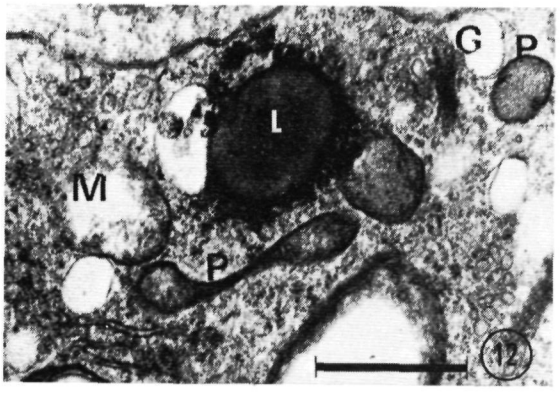
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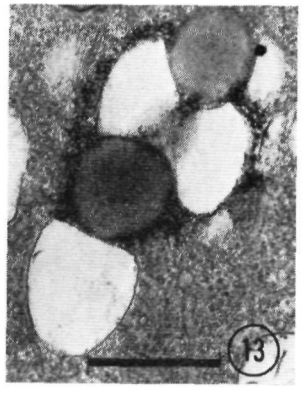
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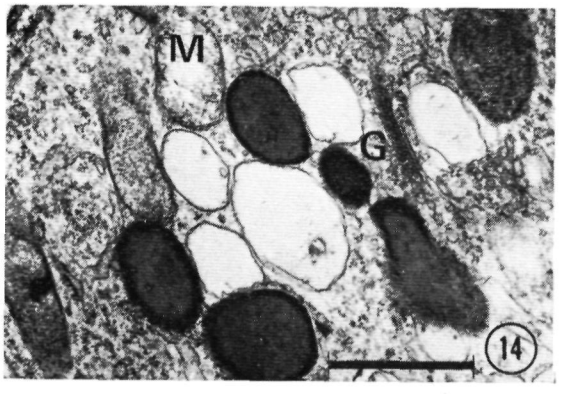
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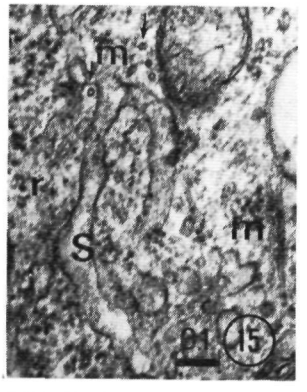
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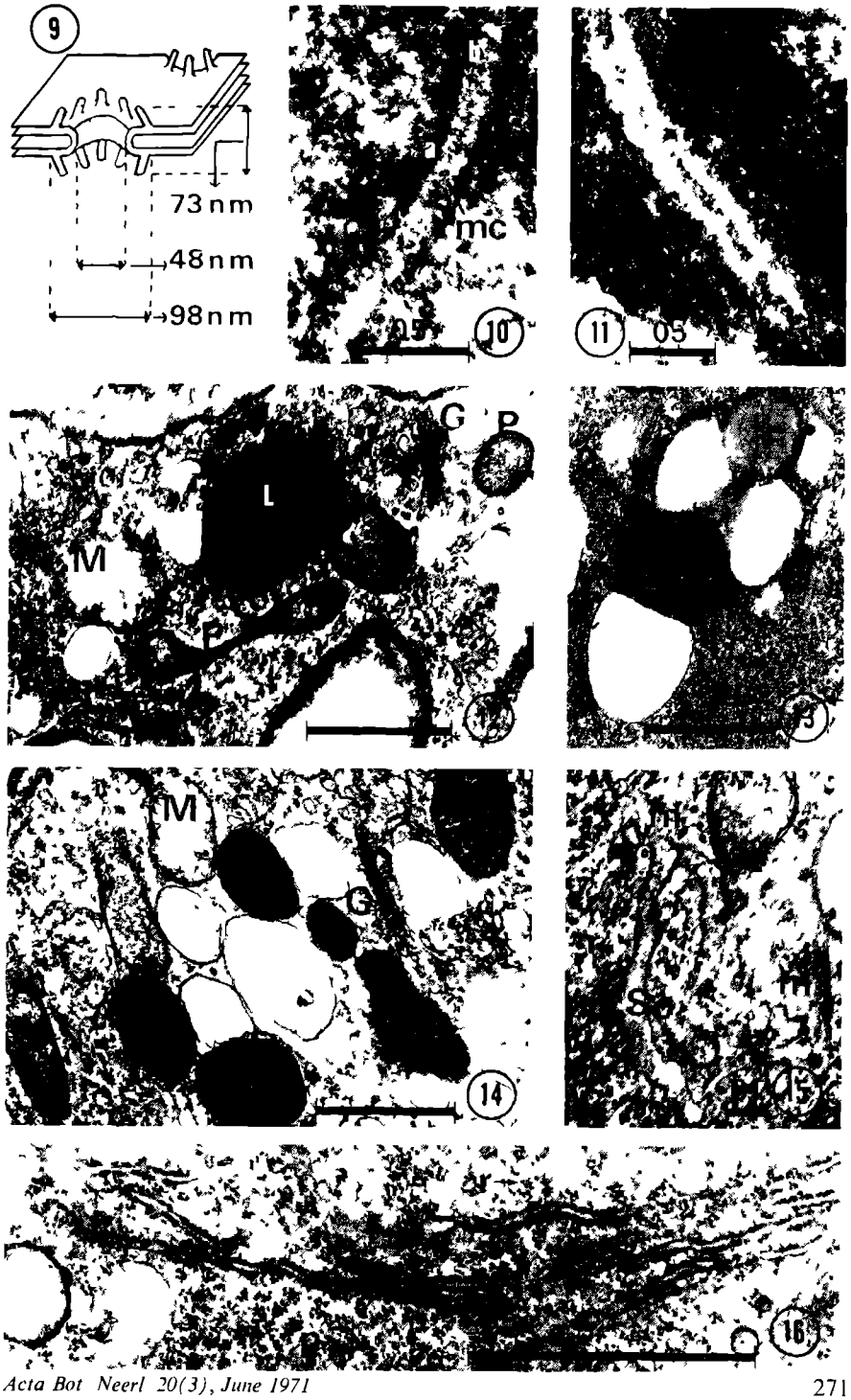
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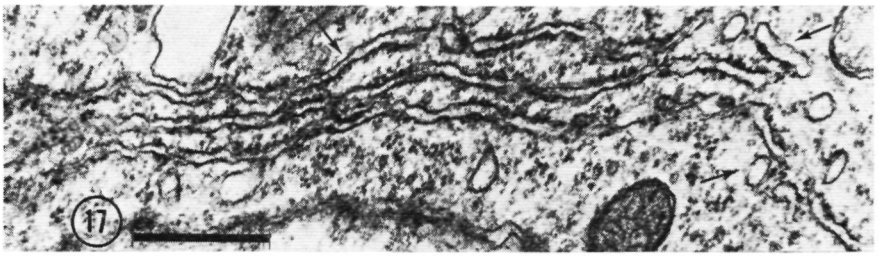
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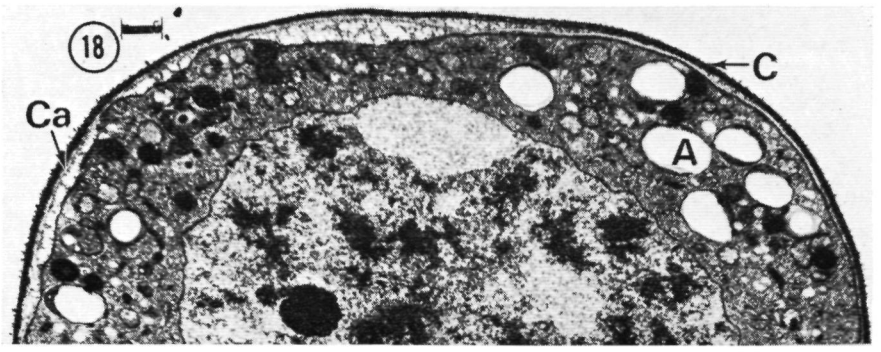
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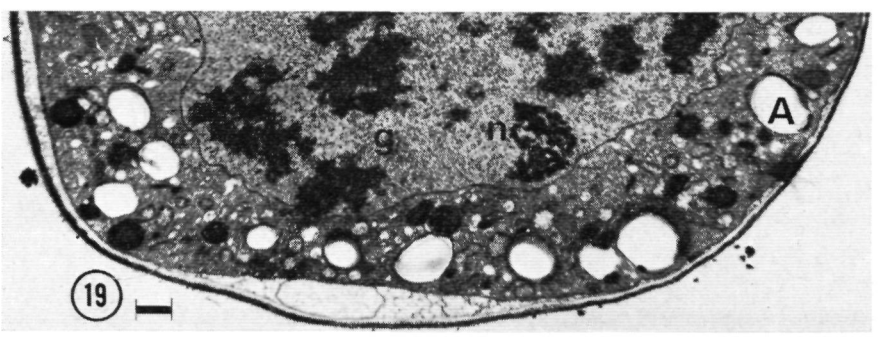
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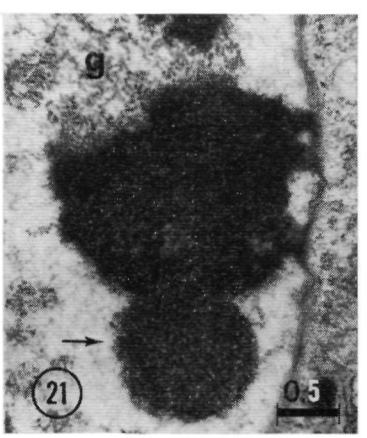
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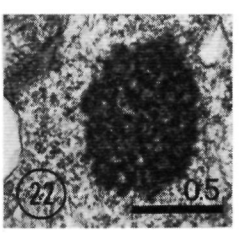
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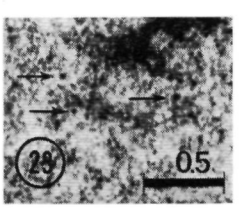
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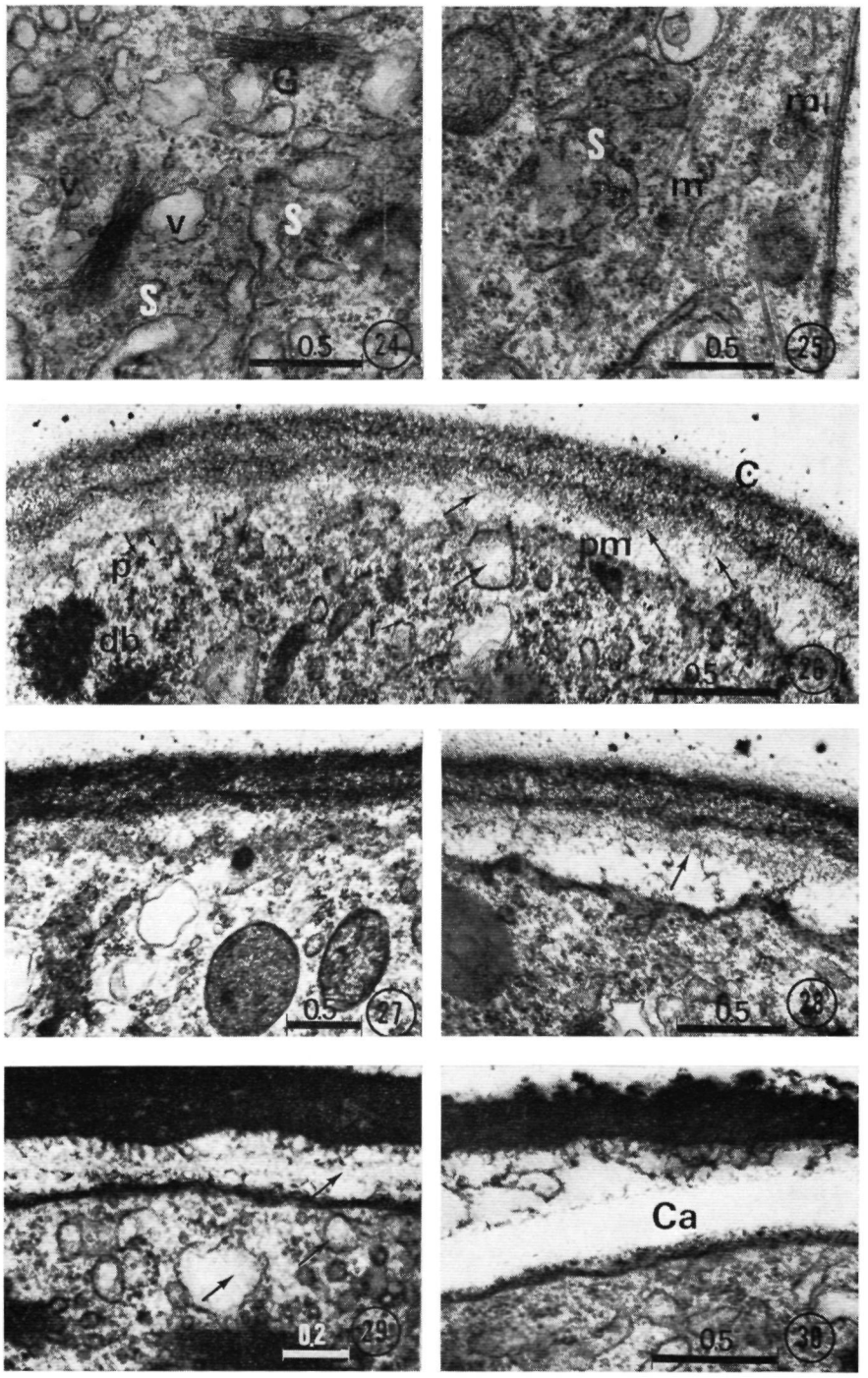
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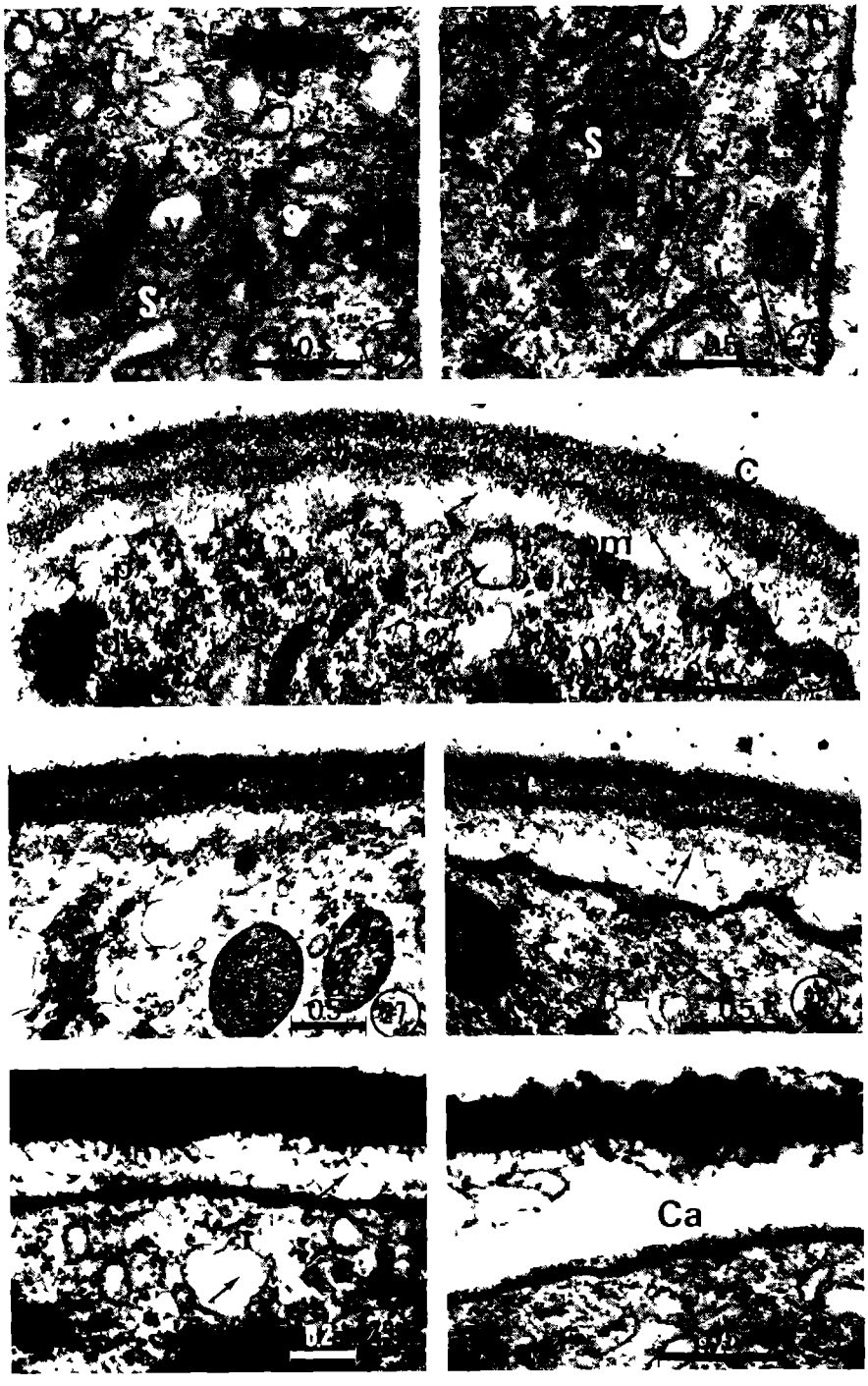
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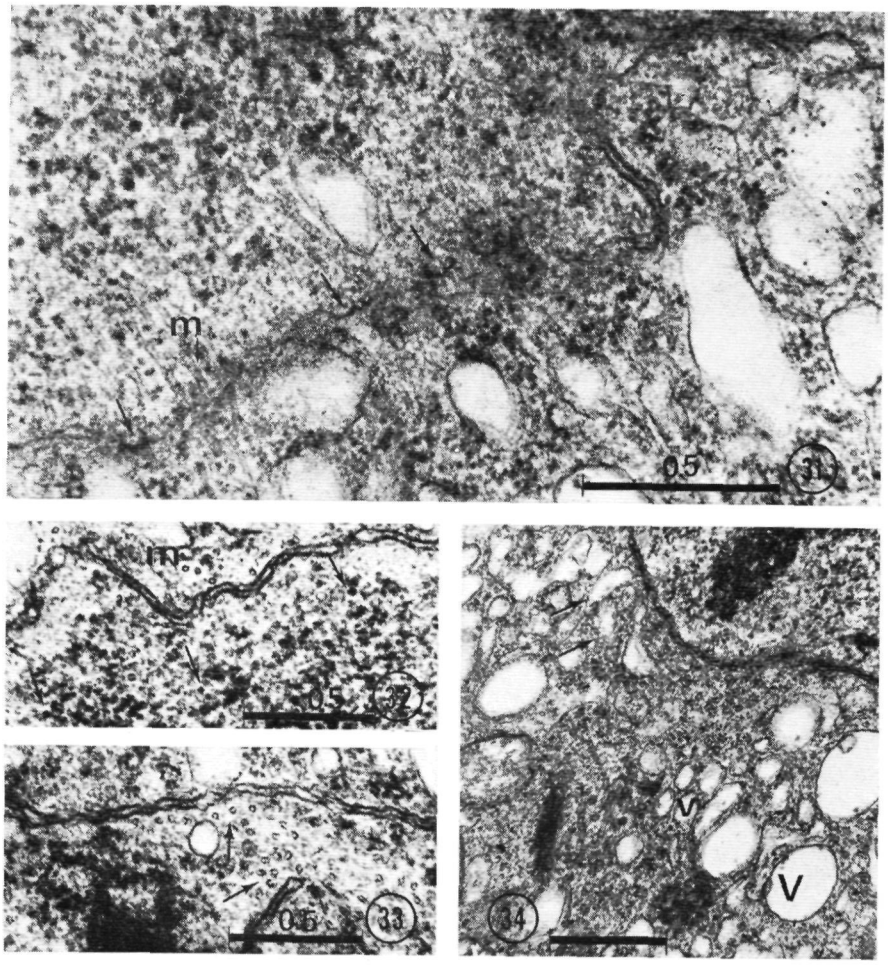
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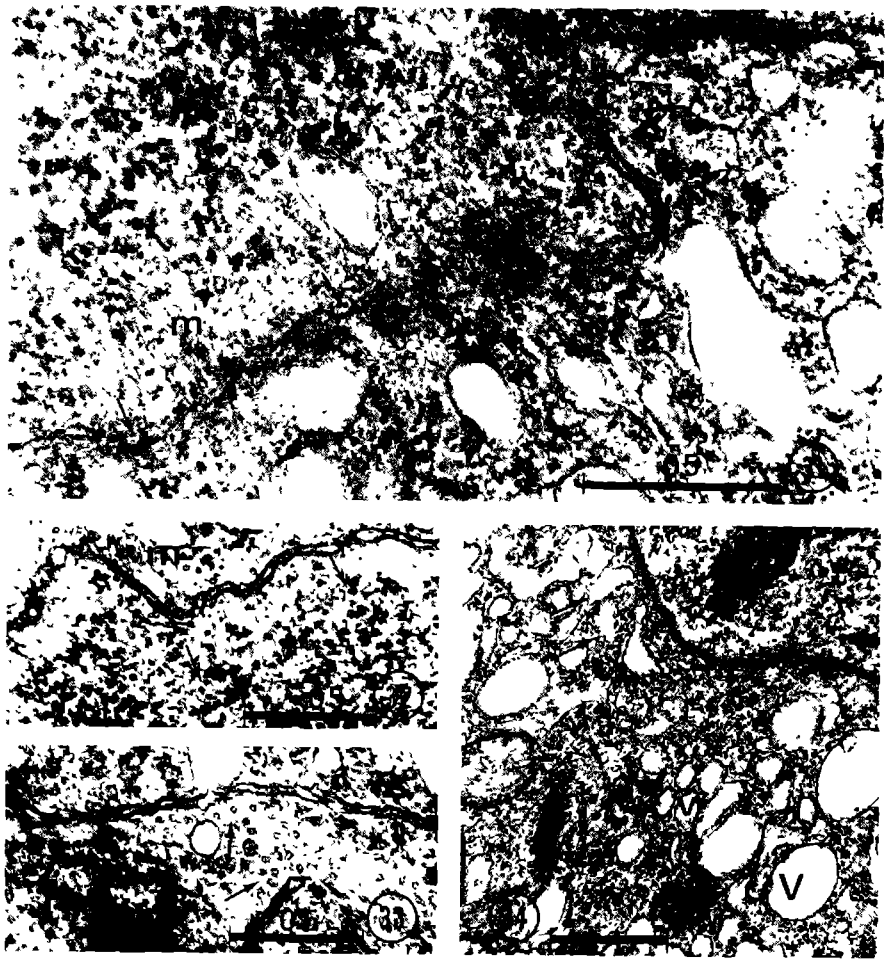
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MORPHOLOGICAL AND QUANTITATIVE CHANGES IN THE POPULATION OF CELL ORGANELLES DURING MICROSPOROGENESIS OF PINUS SYLVESTRIS L. II. MORPHOLOGICAL CHANGES FROM PROMETAPHASE I UNTIL THE TETRAD STAGE

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SUMMARY

A morphological study was made with respect to the changes during microsporogenesis of *Pinus sylvestris* L. from prometaphase I until the tetrad stage. Described and discussed are the breakdown and rebuilding of the nuclear membrane, the mixing of karyoplasm and cytoplasm with the transfer of ribosomal material, the nuclear invaginations, the kinetochore and microtubuli.

A decrease of starch in the plastids starts in interphase II. The mitochondria do not change. The "lipid-complex" appears in telophase II. Many vesicles are present in the cytoplasm during the cell divisions. Ribosomes are observed in all stages, polysomes become visible during interphase II and telophase II. Rough endoplasmic reticulum is absent. The Golgi body produces small and large vesicles and long flat cisternae, similar to smooth endoplasmic reticulum. These Golgi vesicles fuse and form the primary cell wall. The presence of the plasma membrane is postulated as necessary for the synthesis of callose. No difference has been found between the morphological events during callose wall formation and cellulose wall formation. A relation may exist between the callose wall formation and the polysomes and lipid granules and "lipid complex", but a relation with smooth endoplasmic reticulum is not clear. During diplotene and early tetrad stage the formation of the callose wall proceeds in the same way.

1 INTRODUCTION

During meiotic cell division in *Pinus sylvestris* many events occur which are similar to those during the mitotic divisions in other organisms.

No consensus of opinion exists concerning the breakdown and the rebuilding of the nuclear membrane (ESAU & GILL 1969). The centriole in the plant cell shows the same variability. A polar centre may sometimes be absent (TANAKA 1970) or it may appear as a polar body (ESAU & GILL 1969), or a "polar hollow" (BURGESS 1970), or a vesicle aggregate (ROBBINS & JENTZSCH 1969). The kinetochore during metaphase I in *Tradescantia* (WILSON 1968) and in *Lilium candidum* (DIETRICH 1968) has the same morphological structure as has been found in pine. Nuclear invaginations, as appear in pine during the early tetrad stage, have already been reported in *Pinus banksiana* by DICKINSON & BELL (1970) and for *Podocarpus macrophyllus* by ALDRICH & VASIL (1970). After the breakdown of the nuclear membrane a mixing of the karyoplasm and cytoplasm takes place

In *Lilium* the ribosome population increases mainly during diakinesis and metaphase I (MACKENZIE, HESLOP-HARRISON & DICKINSON 1967; LINSKENS & SCHRAUWEN 1968 and DICKINSON & HESLOP-HARRISON 1970). Transfer of ribosomal material from the nucleus to the cytoplasm during mitosis in *Vicia faba* is supposed by LAFONTAINE & CHOUINARD (1963) and in *Pinus sylvestris* during meiosis by WILLEMSE & LINSKENS (1968). MARUYAMA (1965, 1968) found no change in plastids, mitochondria and Golgi bodies during the meiotic divisions of *Tradescantia paludosa*. No change in mitochondria and Golgi bodies occurs during mitosis (ROTH, WILSON & CHAKRABORTY 1966).

During the early tetrad stage callose wall formation takes place. MARTENS, WATERKEYN & HUYSKENS (1967) showed that a callose wall surrounds the whole microspore of *Pinus sylvestris* during the tetrad stage. Some studies are known about the callose wall formation between the generative and vegetative cell of the microspore. In *Helleborus foetidus* the earliest wall separating the microspore is formed by aggregation of Golgi vesicles, which contain callose (ECHLIN & GODWIN 1968). In *Dactylorchis fuchsii* and *D. purpurella* the vesicles forming the cell-plate contain callose or callose precursors (HESLOP-HARRISON 1968). During the formation of the generative cell wall in *Endymion non-scriptus*, vesicles, microtubules and tubular endoplasmic reticulum (ER) are present. It has been suggested by ANGOLD (1968), that the structure of the generative cell wall contains callose, because tubular ER is present. TSINGER & PETROVSKAYA-BARANOVA (1965) report that sphaerosomes are connected with the formation of callose plugs in pollen tubes of *Lathyrus odoratus*.

In cellulose walls the formation of the cell-plate starts in the phragmoplast which consists of microtubules oriented at right angles to the plane of the future cell wall, in which many Golgi vesicles fuse (FREY-WYSSLING *et al.* 1964; NORTHCOTE & PICKETT-HEAPS 1966; PICKETT-HEAPS 1967; HEPLER & JACKSON 1968 and HEPLER & NEWCOMB 1967). In general during the formation of the cellulose wall the Golgi vesicles contain and transport material for the primary cell wall (SIEVERS 1963; SASSEN 1964; JORDAN 1970). NORTHCOTE & PICKETT-HEAPS (1966) supposed that in a separate way cellulose should synthesize and they restrict the function of the Golgi body to the synthesis of pectic substances only. The multiple enzymatical function of the Golgi bodies has been supposed by PICKETT-HEAPS (1967) and demonstrated by WISE & FLICKINGER (1970). In the cisternae of the Golgi body many particulate enzymes have been found (WERZ & KELLNER 1970; STAEHELIN & KIERMAYER 1970).

Cellulose formation may take place on the plasma membrane and is not related to a distinct cell organelle. During cellulose formation, D-glucose is transferred to a glucolipid, which acts as a carrier and is translocated outside the cell, where the D-glucose is polymerized to cellulose (HASSID 1969). Chemical intact membrane fragments are necessary for cellulose synthetase activity (PINSKY & ORDIN 1969). Polysaccharide-synthesizing particles are present on the plasma membrane (RAY 1967; VILLEMEZ *et al.* 1968). However, BROWN (1969) suggests that a transport of cellulose by the Golgi vesicles takes place in *Pleurochrysis scherffelii*.

PICKETT-HEAPS (1967), HEPLER & NEWCOMB (1967) and HEPLER & JACKSON (1968) suppose, on the basis of the labeling pattern of applied ^{3}H-glucose and the presence of ER near the cell-plate, that the ER plays a role in cell wall synthesis ER is present where callose is formed at the pore sites of the sieve plate in *Cucurbita* (ESAU *et al* 1962)

In pine, primary cell wall materials are produced in the Golgi body and transported by Golgi vesicles The callose is probably synthesized on the plasma membrane The products necessary for the formation of the callose wall are probably stored in the lipid granules or "lipid complex" and in the plastids

2. MATERIAL AND METHODS

The same material and methods were used as is described in the preceding part of this paper (WILLEMSE 1971).

3. RESULTS

3 1. Prometaphase I until telophase I

During these stages of cell division, the volume of the cell increases. The cell is surrounded by a thin cell wall and a callose wall (*fig 1*)

Fragments of the nuclear membrane are visible on the border between karyoplasm and cytoplasm (*fig 1, 2*) The karyoplasm contains granules with a diameter of approximately 30 nm and 15 nm (*fig 3, 4*) The microtubuli are situated near the chromosomes. During prometaphase the kinetochore is visible as a globular less electron dense structure on the chromosome (*fig 3*). During anaphase I microtubules are attached on the kinetochore, which is more granular (*fig. 4*) The microtubuli between the chromosomes extending from pole to pole are present in anaphase I A centriole or a marked direction point in relation to the microtubules could not be observed On the polar ends of the cell elements similar to smooth ER (SER) are lying together in several distinct groups (*fig. 5*).

Many plastids contain a large starch granule (*fig 1*). The mitochondria have few cristae and an electron transparent content The lipid granules contain an electron dense material The Golgi bodies with few vesicles are situated mainly around the granular zone with the chromosomes (*fig 2*) In the cytoplasm many vesicles of the same type as found during diakinesis are present A small population of ribosomes is situated in the cytoplasm

3.2. Telophase I

In the polar ends of the cell a close contact exists between the somewhat homogeneous mass of chromosomes and the new nuclear membrane (*fig 6*). Remarkable is the presence of many Golgi bodies around the chromosome mass. They produce small vesicles which fuse and locally form a new nuclear membrane (*fig 7*).

In the centre of the cell many granules are present, most of them are approxi-

mately 15 nm in diameter. In the equatorial plane the microtubuli end in an accumulation of small vesicles which seem to represent a club-shaped end of the microtubule (*fig 8, 9*). The number of vesicles in the cytoplasm decreases. Mainly vesicles with a clear membrane remain visible.

3 3. Interphase II

The cell has a rather large volume and at each pole lies a nucleus The callose wall shows thickening on the polar ends and has grown in comparison with prometaphase I (*fig 10*)

The nucleus contains homogeneous nucleoli and sometimes inclusions The karyoplasm has few granules of about 30 nm in diameter

In the cytoplasm the starch granule in the plastids decreases in size (*fig 10*) Golgi bodies, producing few small vesicles, are spread throughout the cytoplasm In the centre of the cell many polysomes, ribosomes and some microtubuli are present (*fig. 11*).

3.4. Prometaphase II until the early tetrad stage

The breakdown of the nuclear envelope starts locally with the appearance of dilatations between the two membranes, followed by the fading of the two membranes (*fig. 12*) Here the breakdown initiates at the pole The karyoplasm has many granules of approximately 15 nm in diameter (*fig. 13*). In metaphase II many microtubules show in cross section a crescent form (*fig 14*) A centriole or a direction point has not been observed Vesicles and elements of SER or similar to SER are found only in these regions (*fig. 13*).

The decrease of starch in the plastids continues Some fragments of membranes become visible around the starch granule. Plastids without starch have sometimes a bowed appearance The lipid granules contain an electron dense material During telophase II lipid granules are connected with electron transparent vesicles and the whole group is surrounded by dark dots: the "lipid complex" appears (*fig. 15*). Golgi bodies, producing vesicles, are lying close to the region of the dividing nucleus (*fig 12*). During cell division the cytoplasm contains many vesicles with a clear membrane and dilated cisternae of SER and Golgi vesicles similar to those found during diakinesis Ribosomes including some polysomes are observed mainly around the plastids The number of polysomes increases during telophase II; remarkable are the helical polysomes in this stage (*fig. 16*) During metaphase II and telophase II small concentric membranes are observed in the cytoplasm (*fig. 13, 15*).

3.5. The early tetrad stage

The four nuclei are arranged opposite to each other near the plasma membrane. The nuclei contain homogeneous nucleoli (*fig 17*). Vesicles surrounded by two unit membranes, possibly an invagination of the nuclear membrane, occupy the periphery of the nucleus These vesicles contain thin fibrillar material and are surrounded with karyoplasm or chromatin material (*fig 18*).

In the cytoplasm the decrease of starch in the plastids continues. The lipid

granules have a different electron density, the "lipid complex" is also present The Golgi bodies are dispersed in the cytoplasm (*fig 17*) Many polysomes and ribosomes are present Some electron transparent zones in the cytoplasm contain fibrillar material, resembling thin threads of chromatin, which converts into more electron dense threads (*fig 19, 34*)

3 6 The callose wall formation

The callose wall between the four cells grows in centripetal direction (*fig 33*) First the fusing vesicles of the cell-plate are visible everywhere at the same time (*fig 17*)

During anaphase II and telophase II many irregular shaped vesicles are present near the chromosomes In the early tetrad stage vesicles connected with ribosomes have been observed near the nucleus (*fig 20*) The ribosomes on the vesicles disappear and the result is a very dilated vesicle or cisterna, similar to SER Except the large vesicles, many small irregular shaped vesicles, small round vesicles and long flat cisternae are present in the early tetrad stage (*fig 21*) The long flat cisternae look like SER However, the Golgi bodies produce small round vesicles, large vesicles as well as long flat cisternae (*fig 22, 23*) The long flat cisternae in the cytoplasm and in the vicinity of the future cell-plate, looking like SER, originate from Golgi bodies Many Golgi vesicles and long flat Golgi cisternae are situated in the region of the future cell-plate (*fig 24*) These Golgi cisternae and vesicles fuse by making contact with their membranes The two layers of the unit membrane become now clearly distinguishable (*fig 25, 26, 27*). In the places where the membranes touch each other, sometimes some remnants of these membranes remain visible in the vesicles (*fig 30*)

The fused vesicles are the first elements for the cell-plate (*fig* 26) A very thin line in the direction of the cell-plate becomes now visible in the somewhat electron transparent centre of the vesicles (*fig, 25, 27*) The content becomes then more electron transparent, the volume increases and in the centre of the vesicle fine fibrillar material appears in the plane of the future cell-plate (*fig 28*) The fine fibrillar material disappears in the electron transparent content of the large vesicle (*fig 29, 30*) Thereafter the callose becomes visible as a very electron transparent line (*fig 31, 32*) Remarkable is the persistence of lead-containing particles mainly near the membrane of the callose containing vesicles and near the callose wall, which surrounds the whole cell (*fig 21, 30*)

During the cell-plate formation microtubules are found perpendicularly to the fusing vesicles (*fig 34*)

4 DISCUSSION AND CONCLUSION

4 1 The nucleus

A local breakdown, possibly enzymatical, of the nuclear membrane in diakinese and prometaphase II starts at the pole, which has also been reported for *Haemanthus* (BAJER & MOLF-BAJER 1969) Remnants of the nuclear membrane remain visible in the cytoplasm After diplotene no rough ER (RER), but only short

cisternae of SER are present, therefore the membrane bundles, which are visible during the division stages, have to be considered as remnants of the nuclear membrane. In pine the new nuclear envelope may be formed from the remnants of the nuclear membranes and of Golgi vesicles, which fuse to rebuild partly the lacking nuclear membrane parts. Many Golgi bodies surround the telophase nucleus, while also fusing vesicles, forming a new nuclear membrane, are present. In HeLa cells ROBBINS & JENTSCH (1969) supposed the re-formation of the nuclear membrane from spheroids derived from polar vesicular aggregates. Fusion of vesicles and cisternae of the ER have also been described as to reform the nuclear membrane (ESAU & GILL 1969). The capacity of the Golgi vesicles to rebuild the plasma membrane is shown by the formation of the cell-plate in *Phaseolus* roots (HELPER & NEWCOMB 1967) or in pine, as is described here, and in the cell coat of several types of rat cells (RAMBOURG *et al.* 1969). In pine the nuclear membrane originates also partly from fusing Golgi vesicles.

During prometaphase I the kinetochore in pine is visible as a less electron dense structure on the chromosomes as has been observed in *Tradescantia* (WILSON 1968) and *Lilium* (DIETRICH 1968). In metaphase I the microtubuli become visible on the kinetochore. An organized structure resembling a centriole or polar body (ESAU & GILL 1969; BURGESS 1970) has not been observed in pine as in the fungus *Basidiobolus ranarum* (TANAKA 1970). As WILSON (1971) postulates, ER may function as a polar centre. In pine SER is found in the polar ends, but a relation with microtubules is not present.

In interphase II inclusions have been observed in the nucleus. In the early tetrad stage invaginations of the nuclear membrane become visible, similar to those described during the tetrad stage for *Podocarpus* by VASIL & ALDRICH (1970) and for *Pinus* by DICKINSON & BELL (1970). In *Podocarpus* the invaginations may function as channels for the transport of some exine precursors which are synthetized within the nucleus. In *Pinus* the invaginations contain and transport probably nucleic acids to the cytoplasm. In *Pinus sylvestris* the vesicles are probably also invaginations. If there is a transport of material out of the nucleus the thin fibrillar material present in electron transparent zones in the cytoplasm of *Pinus sylvestris* may possibly be delivered from the nucleus by means of the invaginations. This material possibly plays a role in the pollen wall formation which starts after the early tetrad stage. However, a direct relation has not been demonstrated clearly.

After breakdown of the nuclear envelope, the granules in the karyoplasm are mixed in the cytoplasm and not included again in the new nuclei. During diplotene granules of approximately 30 nm, probably ribonucleoproteins, and 15 nm originate from nucleoli. The 15 nm granules strongly resemble ribosomes. Many polysomes become visible during interphase II and telophase II after mixture with the cytoplasm in the cell centre. LAFONTAINE & CHOUINARD (1963) described in mitotic cells of *Vicia faba* a nucleolar production of granules and a mixture of these karyoplasmic granules with the cytoplasm, which have a strong resemblance with ribosomes. ESAU & GILL (1969) show the presence of ribosomes in the spindle of dividing mesophyll cells of *Nicotiana* and suggest an

entry of ribosomes in the nuclear region In pine the karyoplasmic granules are precursors of ribosomes They become clearly visible in the diplotene nucleus and are not included in the telophase I nucleus A rise in the number of ribosomes during metaphase I in *Lilium* meiocytes has been reported by DICKINSON & HESLOP-HARRISON (1970) and LINSKENS & SCHRAUWEN (1968), and in *Lilium* and *Trillium* by MACKENZIE *et al* (1967) This outburst of ribosomes derived from the nucleus during the meiotic cell divisions is a way to provide the cytoplasm with new nuclear information (WILLEMSE & LINSKENS 1968) This phenomenon may not be restricted to the meiotic division only

During prometaphase I and II some microtubuli have mainly a crescent form These structures are the C-filaments like those found in the ciliate *Nassula* (TUCKER 1967) These C-filaments are either artefacts or a development stage in the microtubule formation In pine the C-filaments are observed during prometaphase and they are situated mainly in the region of the nucleus They can be interpreted as a stage of microtubule development No links or cross bridges between microtubuli have been observed as reported by WILSON (1969), HEPLER & JACKSON (1968) and KRISHAN & BUCK (1964)

4 2 The cytoplasm

The decrease of the starch content of the plastids starting in interphase II, is in contrast with the unchanging starch content in the plastids of *Tradescantia* The rod-like mitochondria disappear in *Tradescantia*, but in pine both spherical and rod-like mitochondria persist (MARUYAMA 1968) During microsporogenesis in pine the mitochondria have an electron transparent content and few cristae ROTH *et al* (1966) supposed that no changes in the number of mitochondria and Golgi bodies during the mitotic division take place It is possible that during microsporogenesis in pine no changes in the mitochondria occur, but the Golgi body changes in production of vesicles and changes in position in the cell The small concentric membranes found during metaphase II and telophase II may be compared with the multilayered rings in *Tradescantia* microspores, interpreted by MARUYAMA (1966, 1968) as an early Golgi body stage In all stages lipid granules are present An aggregation of lipid granula during absence of the nuclear membrane as in *Lilium* has not been observed (HESLOP-HARRISON & DICKINSON 1967) As in zygotene and pachytene, the "lipid complex" appears again in prometaphase II Many vesicles with an electron transparent content and a clear membrane have been observed in all stages of development Mainly during the division stages many vesicles, mainly Golgi vesicles and cisternae of SER, surround the dividing nuclei A local accumulation of SER as has been found in dividing rat hepatic cells (DOUGHERTY & LEE 1967) could not be observed The RER is absent After the nuclear divisions polysomes are found in the cytoplasm Ribosomes are always present The presence of helical polysomes has also been reported in pollen mother cells of *Ipomoea* (ECHLIN 1965)

4 3 Callose wall formation

Callose wall formation takes place during diplotene and the early tetrad stage

During telophase I and II microtubuli were observed, standing perpendicularly to the plane of the future cell-plate. In telophase I no cell wall is formed and vesicles are absent. The microtubuli, the phragmoplast fibres (BAJER & JENSEN 1969) lie in clusters in amorphous material and end knobs are found like those reported by HEPLER & JACKSON (1968) for *Haemanthus*. In the early tetrad stage of pine, some microtubuli are standing perpendicularly to the callose wall which surrounds the cell. In diplotene no relation between microtubuli and the callose wall formation has been found.

During the formation of the cell-plate many vesicles and SER-like elements appear. These vesicles and the long flat cisternae similar to the SER are produced by the Golgi bodies. The production of small and large Golgi vesicles on one Golgi body has been reported by PICKETT-HEAPS (1967), as well as the transport of the whole cisternae (BROWN 1969). In pine the production of long flat cisternae similar to SER has to be adjointed to the features of the Golgi body. A fuzzy coated membrane of the Golgi vesicles as reported for *Phaseolus* by HEPLER & NEWCOMB (1967) was not observed.

In the plane of the cell-plate the small or large vesicles and long flat cisternae of the Golgi body fuse. The surrounding unit membrane of these Golgi products becomes more accentuated during the fusion. This membrane is the future plasma membrane (HEPLER & NEWCOMB 1967). In pine these accentuations of the unit membrane of the vesicles in the plane of the cell-plate, but not in the cytoplasm or near a Golgi body, give rise to the supposition that this phenomenon is not the result of normal sectioning as HEPLER & NEWCOMB (1967) suggested. The change in the unit membrane of the vesicles may be a sign of the presence of particulate enzymes on these membranes with relation to the synthesis of callose (VILLEMEZ *et al.* 1968; HASSID 1969; RAY 1967). This may be also the reason for the always dark stained membranes of the Golgi body on which particulate enzymes are present (STAEHELIN & KIERMAYER 1970; WERZ & KELLNER 1970). Probably the enzymes are located between the two layers of the unit membrane (WEHRLI *et al.* 1969). In the Golgi vesicles particulate enzymes could be present derived from the Golgi cisternae. If enzymes are present for the synthesis of callose (or cellulose in another case), they have to function on the membranes of fused vesicles or on the plasma membrane, since the appearance of callose starts within fused vesicles and not during the transport. The necessity of the presence of the plasma membrane appears from the further growth of the cell wall after the disappearance of the Golgi vesicles (HEPLER & NEWCOMB 1967) and the contact of the plasma membrane with the callose wall during the formation of the protrusions in the tetrad stage, when the pollen wall formation starts (WILLEMSE 1971a) as will be described in the next part.

In the fused vesicles fine fibrillar material appears in line with the plane of the future cell-plate; thereafter the callose becomes visible first as an electron transparent central region, which subsequently changes into an electron transparent line in the plane of the cell-plate. During diplotene the same phenomena are observed: small and large Golgi vesicles, SER, possibly also the long flat cisternae derived from the Golgi body, then outside the plasma membrane first

the fine fibrillar material and finally the electron transparent line of the callose wall. Callose wall formation depends on the Golgi vesicles containing materials for the primary cell wall and the presence of the plasma membrane. Callose wall formation does not differ from the cellulose wall formation in its morphological description.

In pine the role of the SER is not clear. A transition of RER to SER before diplotene has been described. The vesicles coated with ribosomes in the early tetrad astge, as reported also by ROBBINS & JENTZ (1967) in dividing HeLa cells, may be analogous to the transition from RER to SER, because of the disappearing ribosomes. ER is related to the formation of plasmodesmata (HEPLER & NEWCOMB 1967), to the pore sites of sieve plates (ESAU *et al.* 1962) and SER to the formation of the callose wall of generative cells (ANGOLD 1968) and of the callose wall around the microspore (ANGOLD 1967; HESLOP-HARRISON 1966). In pine the difficulty is to distinguish the SER from the long flat cisternae of the Golgi bodies. Therefore, their relation to the formation of the callose wall remains hypothetical. Remarkable is the presence of polysomes during callose wall formation. Besides the plastids, the "lipid complex" and the lipid granules may be a storage for polysaccharides as TSINGER & PETROVSKAYA (1965) suggested.

The centripetal ingrowth and the polar thickenings of the callose wall may be connected with the high number of Golgi vesicles at the onset, because the volume of cytoplasm is great on the polar ends of the cell and at the border of the cell centre after the divisions. The orientation of the Golgi vesicles may depend on the presence of microtubuli (HEPLER & NEWCOMB 1967). The orientation of the fine fibrillar material around the cell during diplotene and in the Golgi vesicles during the cell-plate formation may depend on the position of the plasma membrane. The pattern of growth of the cell-plate may be regulated by the adjacent cytoplasm as HEPLER & NEWCOMB (1967) suggested.

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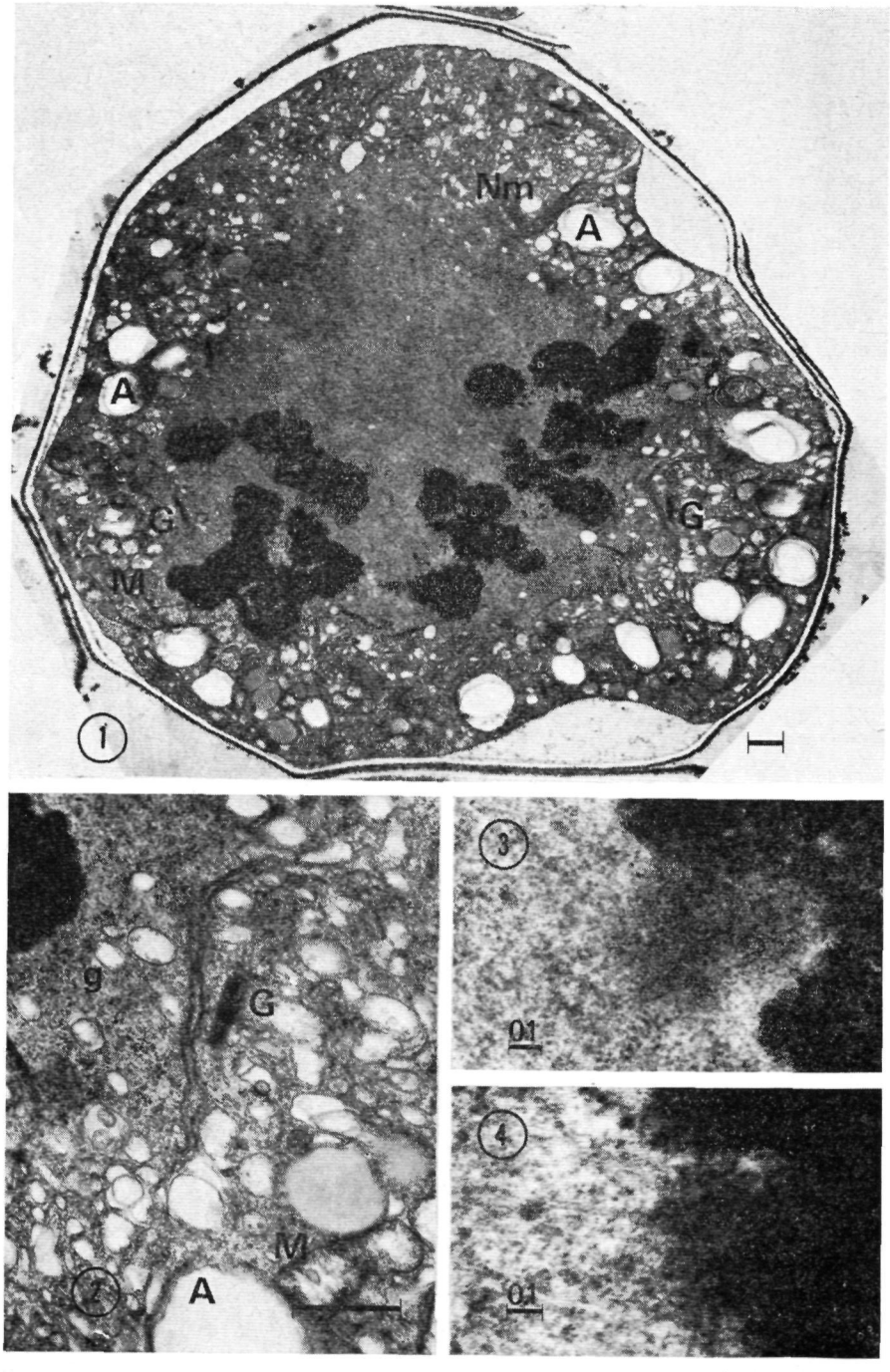
Fig. 1. Prometaphase I. Note the Golgi bodies (G) and remnants of the nuclear membrane (Nm), × 4,770.
Fig. 2. Detail cytoplasm. Golgi bodies (G) round the karyoplasm with many granules (g) Plastids with starch (A) and mitochondria (M), 15,000.
Fig. 3. Kinetochore during prometaphase I. Granular karyoplasm, × 41 000
Fig. 4. Kinetochore during anaphase I with microtubuli. Granular karyoplasm, × 51,800
Fig. 5. Detail cytoplasm anaphase I near the pole of the cell, SER and microtubuli (m), × 21,900.
Fig. 6. Telophase I; rebuilding of the nuclear membrane, many Golgi bodies (G) surround the new nucleus, × 17,400.
Fig. 7. Detail of nuclear membrane (Nm) with Golgi body producing vesicles (v) which fuse (arrow), × 28,800.
Fig. 8. Telophase I; ending microtubuli in the cell centre (arrow), × 21,600.
Fig. 9. Detail microtubule with club-shaped end (arrow), 108,000.
Fig. 10. Interphase II. Cell with callose wall (C) and decreasing starch (A) in the plastids, × 3,060.
Fig. 11. Interphase II. In the cell centre microtubules (m) and polysomes (p), 21,900.
Fig. 12. Prometaphase II Breakdown of the nuclear membrane (Nm). Karyoplasm with granules (g) and Golgi bodies (G), × 24,800
Fig. 13. Anaphase II. Many vesicles (v) around the granular zone. No distinct polar centre. Concentric membranes are present (c), 5,400.
Fig. 14 Microtubules near the chromosomes. Some have a crescent form (arrow), 32 200.
Fig. 15. Telophase II "Lipid complex" in the cytoplasm (Lc). Concentric membranes (c) and decreasing starch (A) in the plastids, × 31,500
Fig. 16. Helical polysomes in the telophase II cell, × 47,300.
Fig. 17. Early tetrad stage. Cell-plate becomes visible between the cells. In the cell light zones with fibrillar material (arrow) Note the "lipid complex" (Lc), 8,640.
Fig. 18. Nuclear invaginations, × 44,400.
Fig. 19. Electron transparent zone with fine fibrillar material, × 43,750.
Fig. 20. Vesicles with ribosomes near the nucleus. Polysomes are visible (p), × 29,400
Fig. 21. Golgi bodies (G) in the cytoplasm with many vesicles (v) and long flat cisternae (arrow), × 21,900.
Fig. 22. Golgi body with small and large vesicle, 35,000.
Fig. 23. Golgi bodies producing long flat cisternae, × 50,600.
Fig. 24. Cell-plate formation. Many Golgi vesicles and cisternae in the cell centre, × 44,400.
Fig. 25. Fusing Golgi vesicles and cisternae, the membrane becomes clearer. Within the vesicles a thin line becomes visible (arrow), × 39,900.
Fig. 26. Fused vesicles with a clear membrane, × 39,900.
Fig. 27. The thin line is visible in the vesicles, × 48,900
Fig. 28. Detail of fused vesicle with more electron transparent content and fine fibrils in plane of the future cell-plate, 36,800.
Fig. 29. The content of the vesicles becomes more electron transparent. Note the black, lead containing particles, × 21,900.
Fig. 30. Detail of the vesicles, in the vesicles remnants of membranes are visible, × 46,000.
Fig 31. In the vesicles the callose becomes visible (arrow), × 18,900.
Fig. 32. Detail of the callose containing vesicle, × 47,500.
Fig. 33. Centripetal ingrowth of the callose wall Note the Golgi bodies (G), 14,700.
Fig 34. Microtubules perpendicular to the coming cell-plate (m). Note the electron dense threads (arrow), × 22,000.

Unless mentioned otherwise, the line in the figures represents a length of 1 μm.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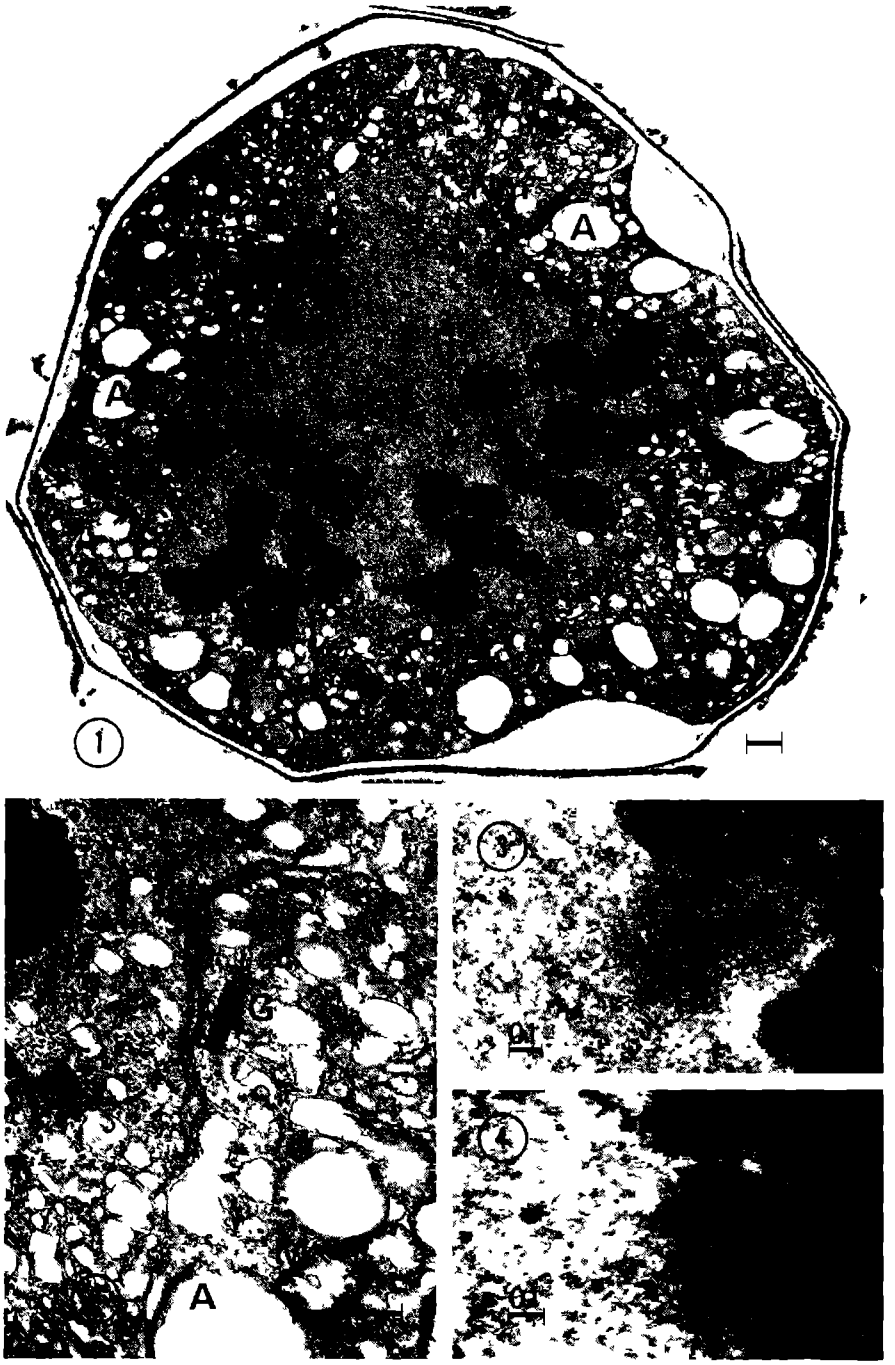
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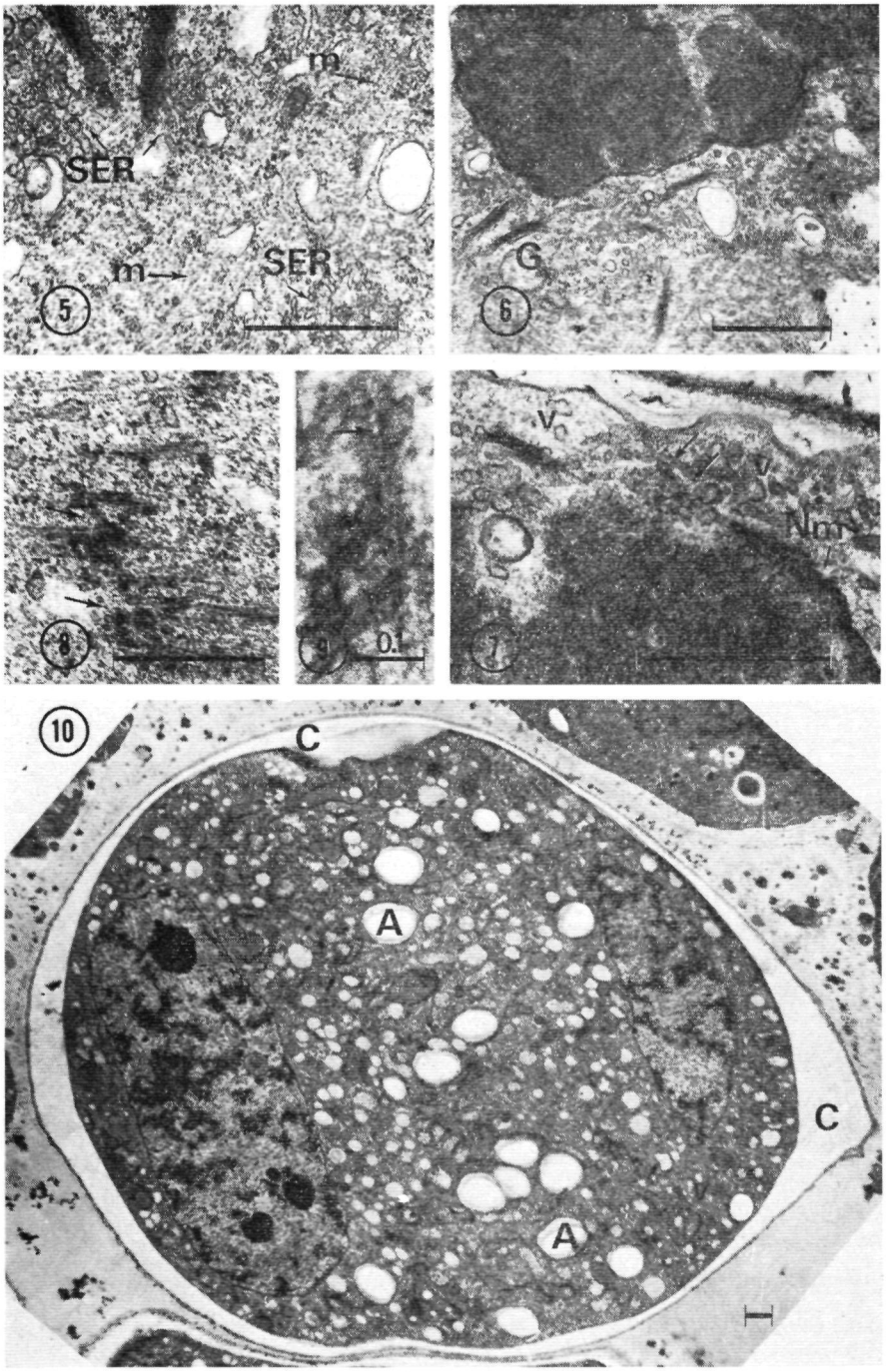
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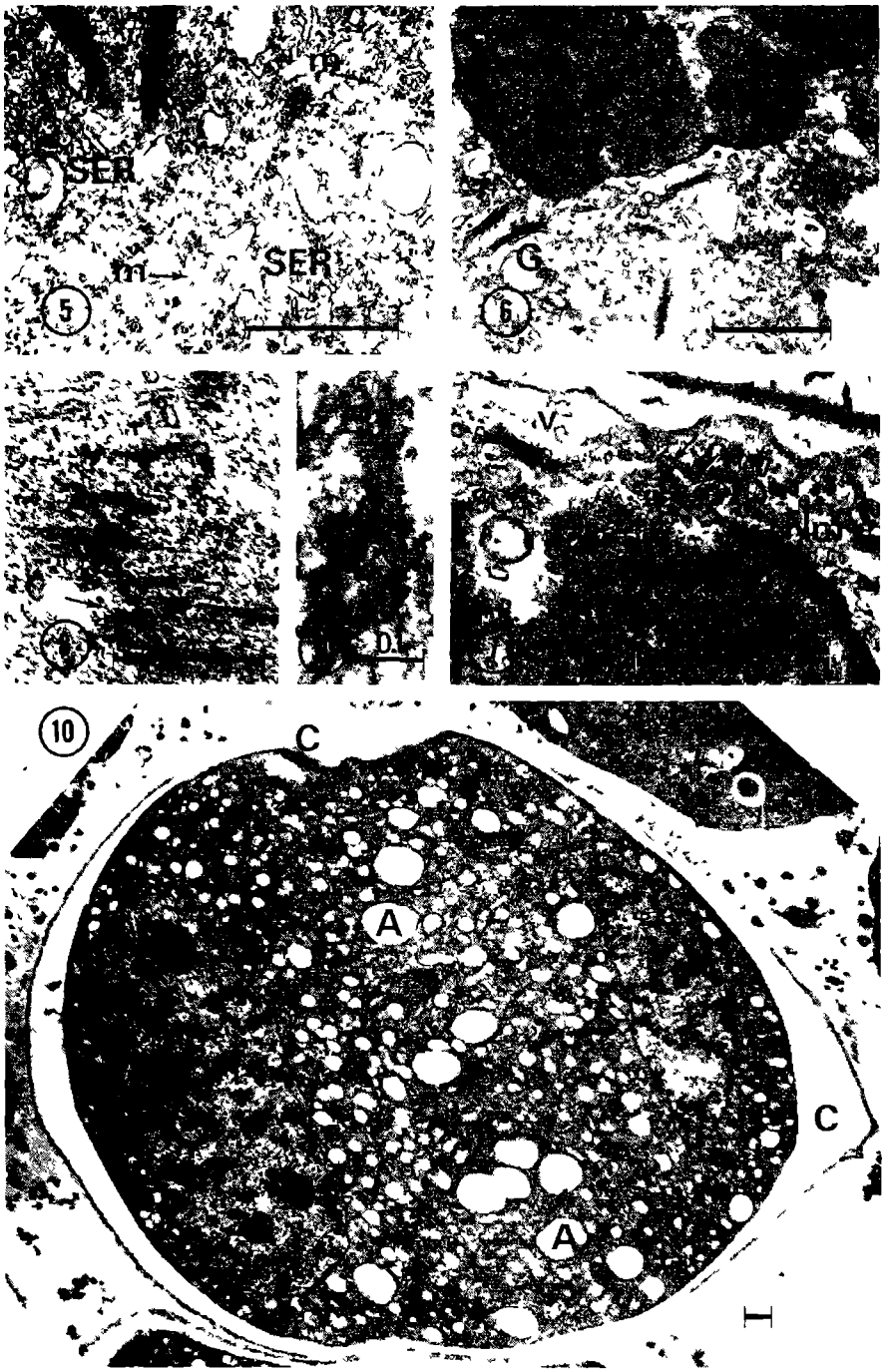
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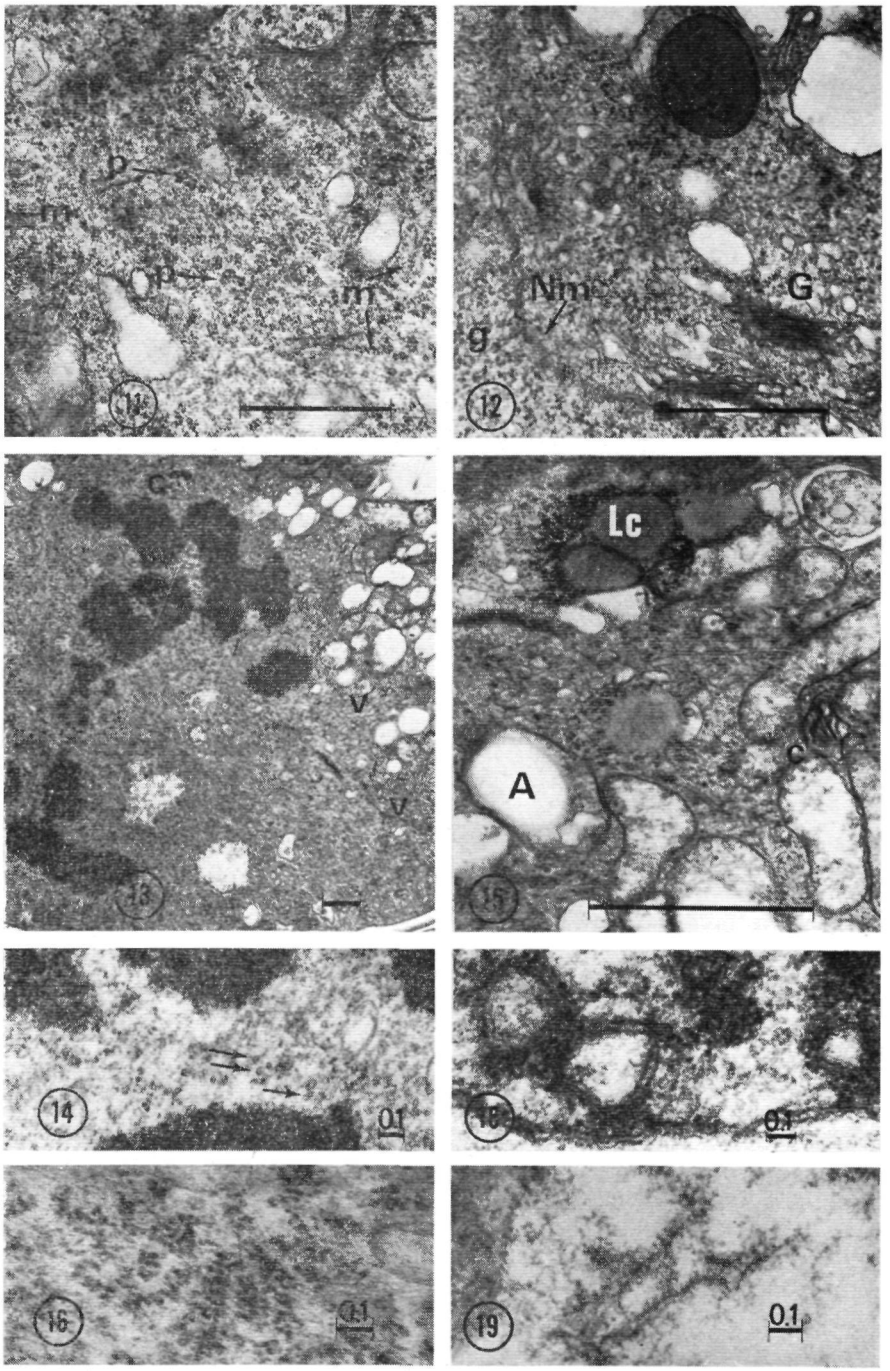
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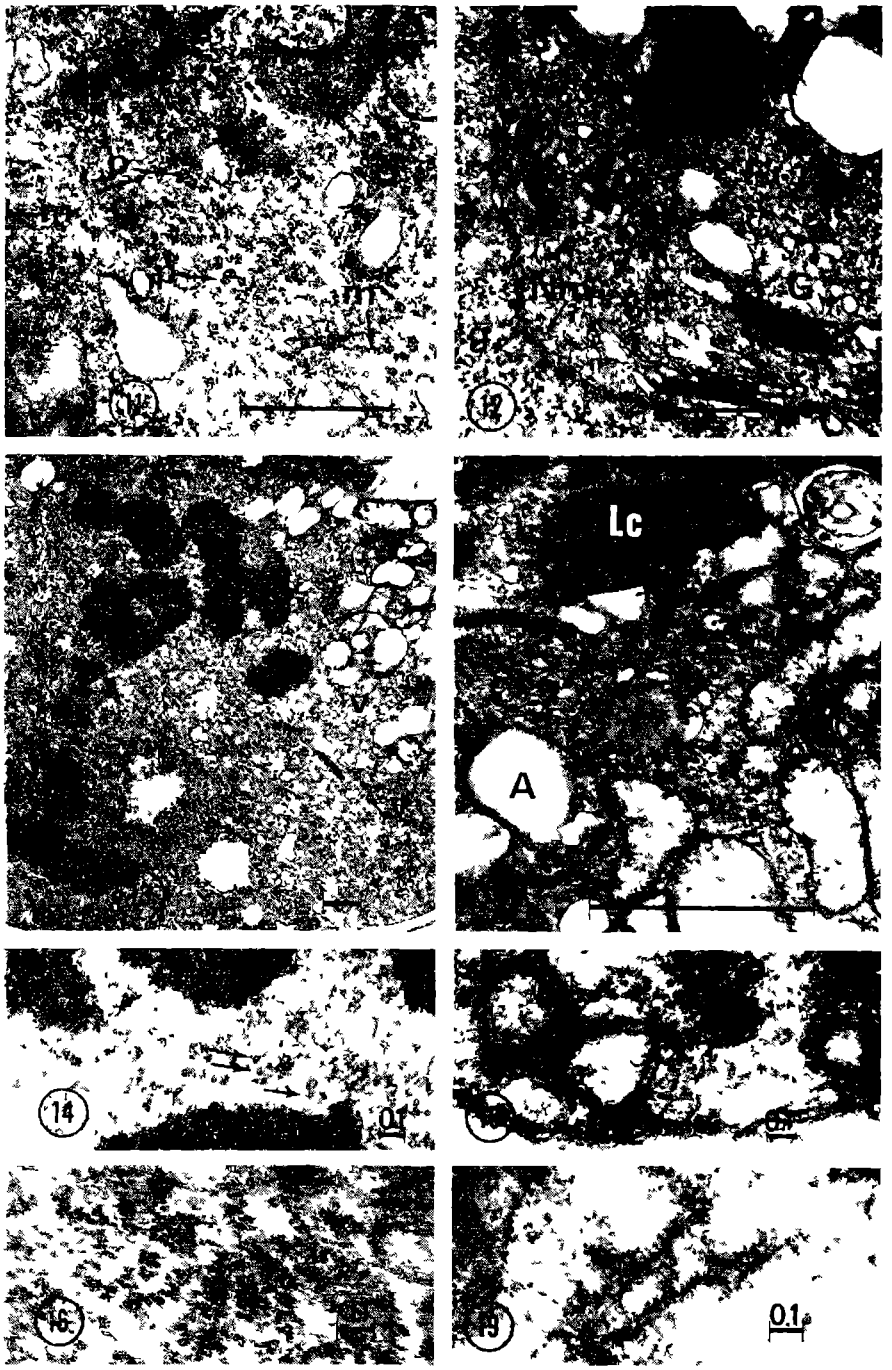
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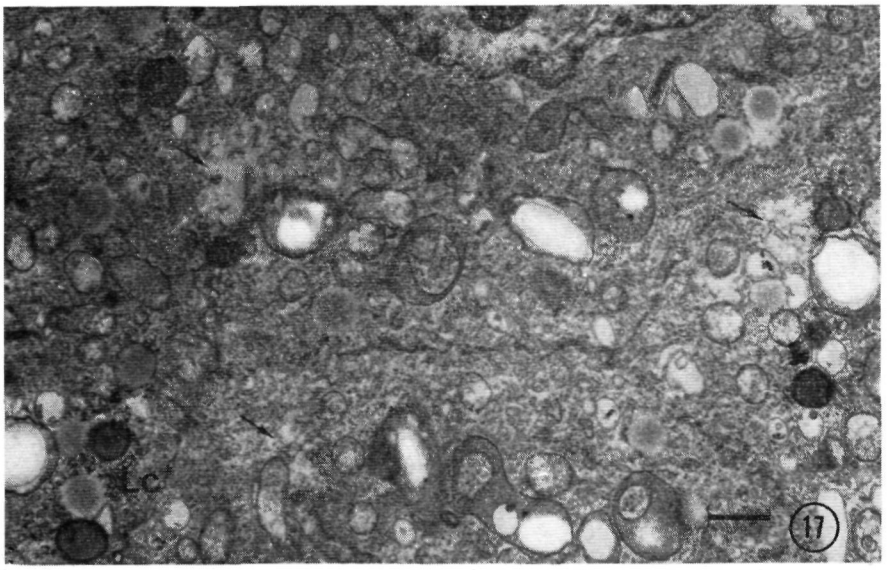
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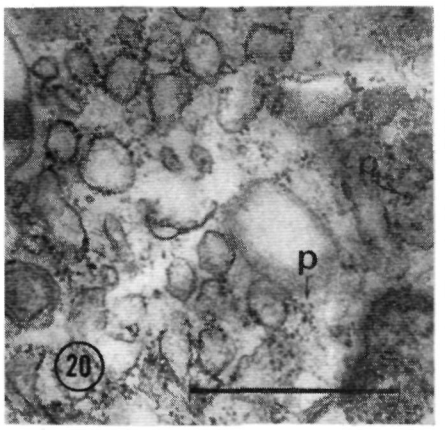
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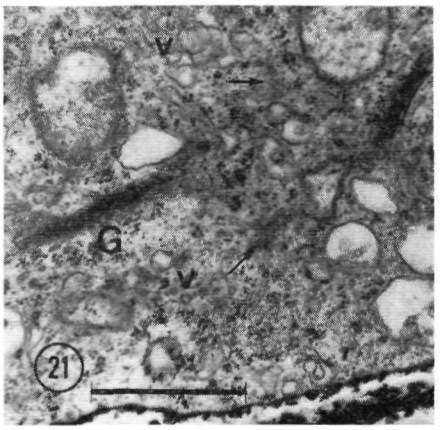
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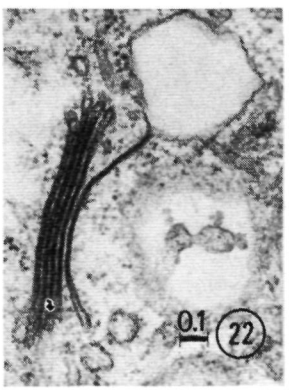
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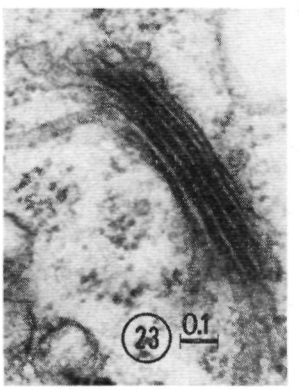
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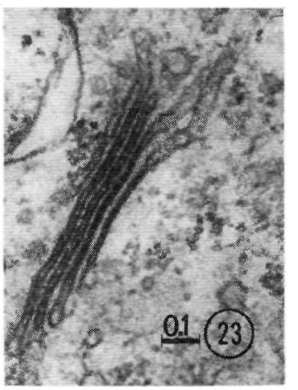
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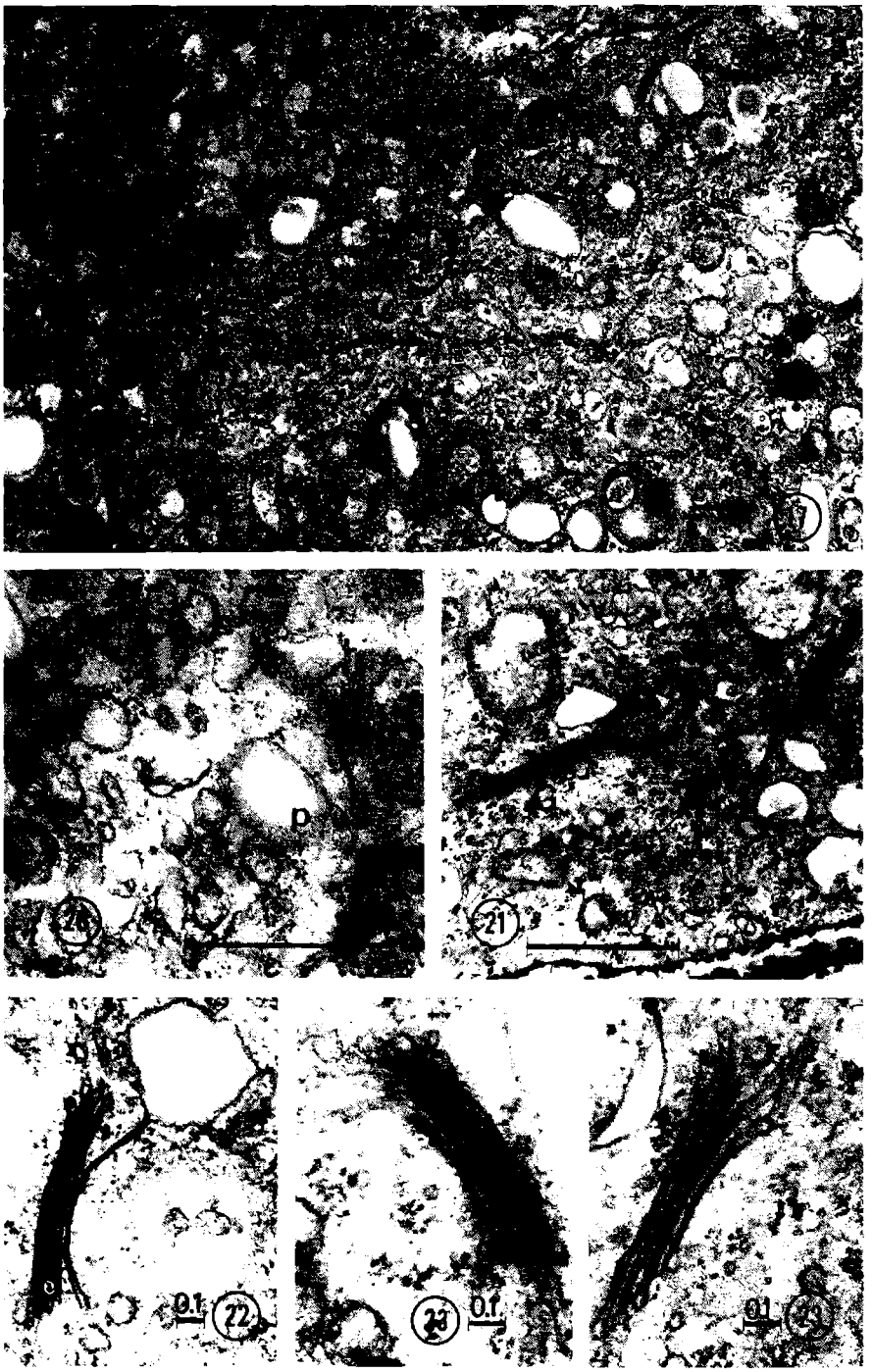
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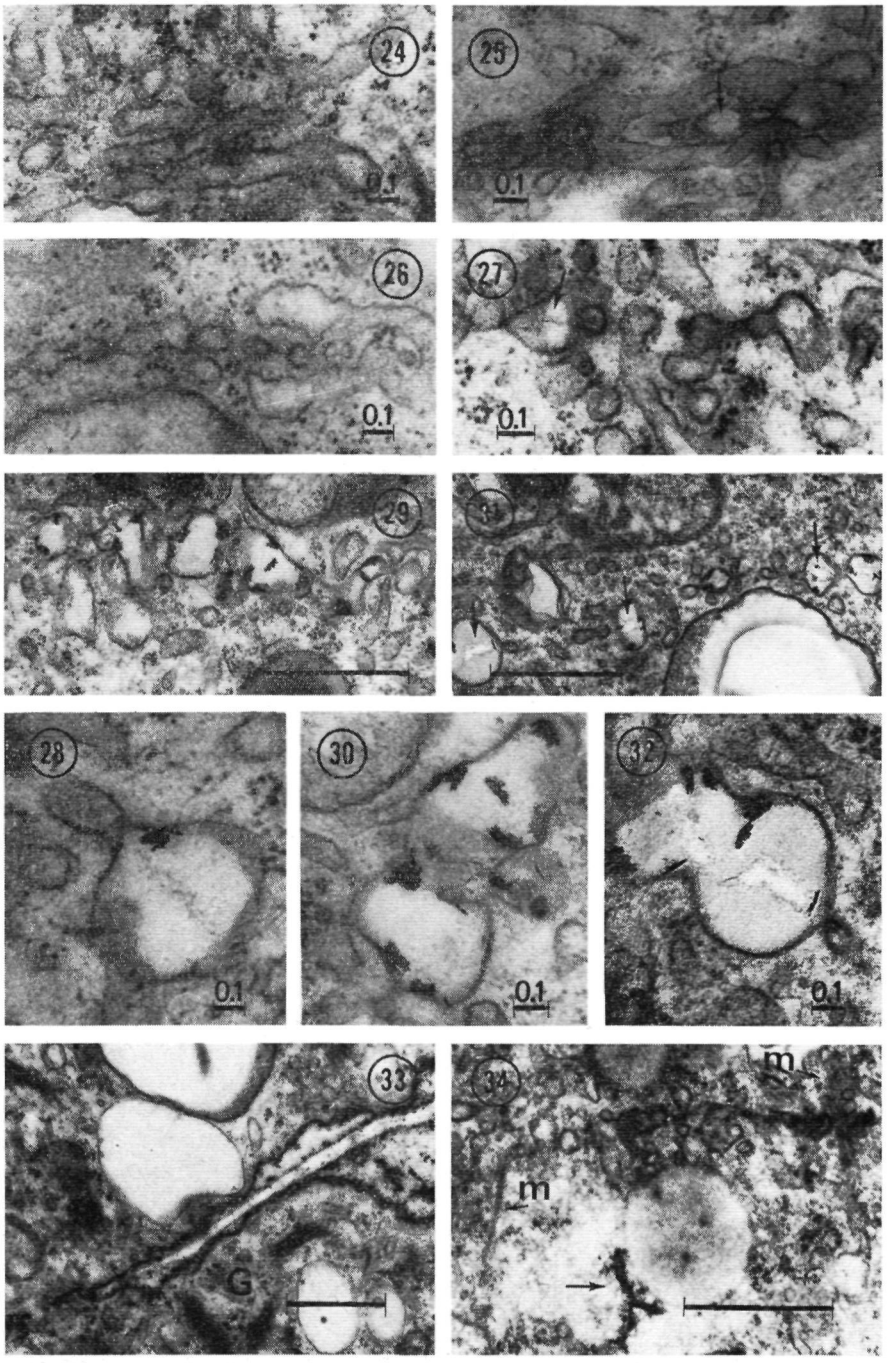
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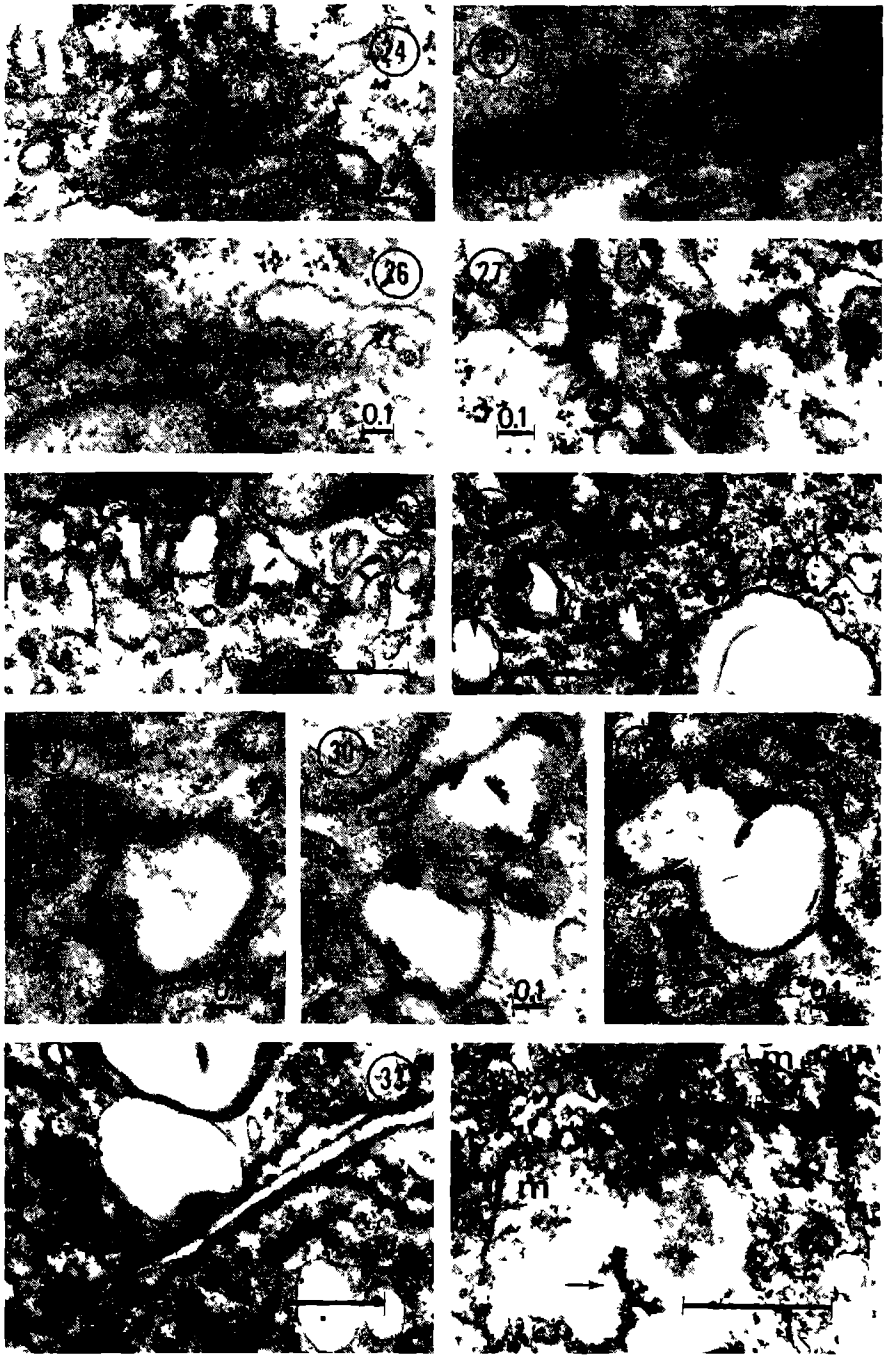
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MORPHOLOGICAL AND QUANTITATIVE CHANGES IN THE POPULATION OF CELL ORGANELLES DURING MICROSPOROGENESIS OF PINUS SYLVESTRIS L.
III. MORPHOLOGICAL CHANGES DURING THE TETRAD STAGE AND IN THE YOUNG MICROSPORE. A QUANTITATIVE APPROACH TO THE CHANGES IN THE POPULATION OF CELL ORGANELLES.

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SUMMARY

A description and discussion are given of the morphological changes of the cell organelles during the middle and late tetrad stage and the young microspore of *Pinus sylvestris* with special reference to the pollen wall formation.

In the middle tetrad stage the template of the pollen wall pattern appears to be the result of Golgi material excretion and callose wall formation. The fine fibrillar material from the content of the Golgi vesicles, consisting partly of a polysaccharide, forms the primesexine. During the late tetrad stage the sporopollenin originates from the microspore and is deposited on membranes mainly outside the cell. It penetrates into the primesexine. The starch in the plastids disappears and the lipid granules become voluminous; both elements are related to the pollen wall formation. The swelling of the sacci may be an osmotic process.

From the quantitative approach it appears, that the young microspore contains approximately one fourth of the number of cell organelles present in the cell during zygotene. The cell organelle population probably does not change in number during the meiotic stages.

A general survey summarizes the whole process of microsporogenesis till the young microspore stage. The control of pollen wall formation is also discussed.

1. INTRODUCTION

1.1. The pollen wall formation

The pollen wall formation takes place during the tetrad stage. The variability and specificity of the pollen wall have evoked many studies about its formation, patterning and chemical composition. Answers to questions about the control and origin of the pollen wall pattern, the formation of sporopollenin and its attachment to the microspore, have been proposed by various authors in different ways.

Although a sporophytic control (ROGERS & HARRIS 1969; MEPHAM 1970; FORD 1971) as well as a gametophytic control (LINSKENS 1969; ECHLIN 1971) have been suggested in the formation of the pollen wall pattern, this control is still questionable. With regard to this point the connection between cytoplasm

and the microspore wall pattern has been reported by HORNER, LERSTEN & BOWEN (1966), ROGERS & HARRIS (1969) and HESLOP-HARRISON (1971). FORD (1971) has concluded that the initiators for the elaboration of the exine pattern have to be present in the cytoplasm before the cell plate formation takes place.

Pollen wall formation starts within the tetrad which is surrounded by a special callose wall. A space between the plasma membrane and the callose wall appears. Thereby a retraction of the plasma membrane (LARSON & LEWIS 1962; SKVARLA & LARSON 1966; LEPOUSÉ 1971) or an extrusion of vesicles outside the plasma membrane have been supposed (ECHLIN & GODWIN 1968). The plasma membrane also shows many undulations or protrusions (SKVARLA & LARSON 1966; ECHLIN & GODWIN 1968; DICKINSON 1970). In the space fibrous material, the primexine appears consisting of cellulose (HESLOP-HARRISON 1968, 1968a). It seems reasonable to assume that it is derived from Golgi vesicles (ECHLIN & GODWIN 1968; DICKINSON 1970). LEPOUSÉ (1970) has demonstrated that Golgi vesicles in the tetrad of *Abies pinsapo* contain polysaccharides and excrete their content. Around the microspore and in the sacci of *Pinus banksiana* periodic acid Schiff positive (PAS +) material is present (DICKINSON & BELL 1970). First the probacula appear in the gaps of the primexine and often above a fold of the plasma membrane, followed by the elements of the tectum. Some relations between the appearance of the probacula and different types of cell organelles were described. A relation with endoplasmic reticulum (ER) has been supposed by HESLOP-HARRISON (1963) and SKVARLA & LARSON (1966); with mitochondria by VAZART (1970); with ribosomes and vesicular components probably derived from Golgi bodies, by HESLOP-HARRISON (1968); with microtubules by ECHLIN & GODWIN (1968); with Golgi vesicles directed by microtubules by DICKINSON (1970) and in *Pinus banksiana* by the position of large cytoplasmic vesicles at the cell surface (DICKINSON 1971).

During the formation of the probacula the callose wall of *Lilium longiflorum* does not change (DICKINSON 1970). In *Abies pinsapo* (LEPOUSÉ 1971) and *Pinus banksiana* (DICKINSON & BELL 1970) long protrusions are visible on the inner side of the callose wall. LEPOUSÉ (1971) showed that these protrusions consist of callose. WATERKEYN & BIENFAIT (1968, 1970) demonstrated a regular geometric pattern on the inner side of the callose wall in *Ipomoea purpurea*, which may be considered as a template for the first spore wall of primexine matrix. In *Styphelia viridis* and *S. triflora* FORD (1971) suggested, that the callose wall is necessary for the ectexine pattern formation.

Sporopollenin is considered to be the main chemical component of the exine. Its first appearance coincides with an increase of electron density of the exine and around the Ubisch bodies or orbicules or plaques in the tapetal fluid (HESLOP-HARRISON & DICKINSON 1969). The electron dense material appears always outside the cell (ECLIN & GODWIN 1968; HESLOP-HARRISON & DICKINSON 1969). The origin of sporopollenin in the microspore has been proved in *Tradescantia bracteata* (MEPHAM & LANE 1970), in the orchid *Eulophidium sandersianum* (CHARDARD 1971), in *Abies pinsapo* (LEPOUSÉ 1971), and has been supposed in *Pinus banksiana* (DICKINSON & BELL 1970) and *Podocarpus macrophyllus* (VASIL

& ALDRICH 1970). On the contrary, it has been supposed that in *Allium cepa* (RISUENO, GIMÉNEZ-MARTIN, LOPEZ-SAEZ & GARCIA 1969) and in *Epidendrum scutella* (COCCUCI & JENSEN 1969) the sporopollenin around the microspore originates from the tapetal cells. Although the origin of sporopollenin remains an open question, the precursors of sporopollenin, possibly without any electron density, have certainly to be synthetized in the microspore as well as in the tapetal cells (HESLOP-HARRISON & DICKINSON 1969). Granules of sporo-pollenin or prosporopollenin have been reported to be present in the cytoplasm of *Eulophidium sandersianum* (CHARDARD 1971). LINSKENS & SUREN (1969) demonstrated that lipid droplets in the cytoplasm of the pollen grain of *Asclepias curassavica* may contribute to the formation of the pollinium wall. More common is the observation of the presence of layers of sporopollenin around the microspore. In these layers on the base of the bacula and tectum, lamellae or laminae of unit membrane dimension have been found (ANGOLD 1967; ROWLEY 1967; LEPOUSÉ & ROMAIN 1967; DUNBAR 1968; ECHLIN & GODWIN 1969; MEPHAM & LANE 1969; HECKMAN 1970; DENIZOT 1971). It has been postulated that the deposition of sporopollenin on lamellae of unit membrane dimension should be a universal mode of sporopollenin deposition (ROWLEY & SOUTHWORTH 1967). Even DICKINSON & HESLOP-HARRISON (1968) extend this deposition of sporopollenin on lamellae to all parts of the exine of *Lilium longiflorum*. Therefore, in theory, the formation of the pollen wall pattern should be related to the plasmalemma and connecting cytoplasmic structures (HESLOP-HARRISON & DICKINSON 1969).

After the breakdown of the callose wall by enzymes in the surrounding tapetal fluid, the microspores are set free (ESCHRICH 1961; MEPHAM & LANE 1969; DICKINSON & BELL 1970). In *Pinus banksiana* the swelling of the sacci may be a result of osmotic forces or imbibition (DICKINSON & BELL 1970).

As mentioned in a previous description (WILLEMSE 1971), the formation of the pollen wall pattern in *Pinus sylvestris* is connected with the excretion of Golgi material simultaneously with a local callose wall formation, at the site of the contact between plasma membrane and callose wall. Within the microspore the sporopollenin originates from membranes. Below the terminology of the pollen wall stratification by ERDTMANN (1969) has been used.

1.2. Quantitative approach

Quantitative microscopical investigations on cell and cell organelles are not used generally (ROSS & JANS 1968), since they are apt to misinterpretation.

From results, obtained by means of a quantitative approach, it seems that in between the cell divisions the plastids divide (MICHAELIS 1962). In *Trifolium hybridum* the plastids are not distributed at random during the cell division (BUTTERFASS 1969). Sphaerosome aggregation and disaggregation during meiosis of *Lilium longiflorum* and *L. henryi* have been demonstrated by a quantitative analysis of electron microscopic photographs (HESLOP-HARRISON & DICKINSON 1967). During the interphase and telophase of meristematic cells of *Epilobium hirsutum* the plastids, mitochondria and Golgi bodies of the whole cell were

counted Just before cell division the Golgi bodies augment, plastids and mitochondria divide during the interphase, while all cell organelles are unevenly distributed (ANTON-LAMPRECHT 1967) During division, changes in cell volume are related to changes in the spindle (BARLOW 1970) The present study deals with a significant change in the number of plastids and Golgi bodies during microsporogenesis of *Pinus sylvestris* An increase of organelles after the meiotic divisions could not be demonstrated

During the sporophytic-gametophytic transition a reorganization of orgaelles in the cytoplasm occurs In *Pinus sylvestris* some of the changes in morphology could be compared with the phenomena, which are also described in mitotic cells From zygotene till young microspore stage the changes in morphology and quantity of the organelles are not so intensive as has been found during microsporogenesis of other plants (HESLOP-HARRISON 1971b)

2 MATERIAL AND METHODS

2 1 Morphological investigations

The same material and methods for collecting, fixation, staining and embedding were used as is described previously (WILLEMSE 1971a, 1971b)

For freeze-etching the microspores of *Pinus sylvestris* were slowly centrifuged in water and a drop of water concentrated with microspores from the pellet was placed on a small copper disc, immediately frozen in liquid freon 22 and subsequently put in the liquid nitrogen With a Balzers apparatus freeze-etching was carried out according to the procedures described by MOOR, MUHLETHALER, WALDNER & FREY-WYSSLING (1961) and MOOR (1964)

2 2 Quantitative approach

Up to the young microspore stage the following stages of development can be distinguished 1 early zygotene (EZ), 2 late zygotene (LZ), 3 pachytene (P), 4 diplotene (D), 5 diakinesis (DA), 6 metaphase, anaphase, telophase I (M I), 7 interphase II (I), 8 metaphase, anaphase, telophase II (M II), 9 early tetrad stage (ET), 10 middle tetrad stage (MT), 11 late tetrad stage (LT) The zygotene has been divided in two parts on the basis of the long duration of the stage and the morphology of the nucleus According to different stages of wall formation the tetrad stage has been divided in three parts

Countings were made on electron microscopical (EM) photographs of sections of complete cells For this investigation distinct photographs of cell sections in the different stages of development were selected In the cell section a large part of the nucleus was present Only sections of totally different cells were chosen

In each stage of development the areas of different numbers of cell sections were determined by counting the number of points of a grid with a mutual distance of 4,45 mm covering the section (Test countings have shown that the error in this method is approximately 1 %) This was done on EM photographs at a magnification of 7,200 × Both the total cell area without cell wall and the

area of the cytoplasm were measured in this way Then the mean value and standard deviation of these areas (number of points) were calculated for each stage of development

The areas of the largest section of intact cells were determined on LM photographs at a magnification of 240 × using the grid (test countings have shown that the error in the method used in this case is approximately 5%). Of these areas the mean value and the standard deviation were calculated

From the cell sections of EM photographs at a magnification of 6,000 × the number of plastids with a starch granule, plastids, mitochondria, lipid granules and Golgi bodies were counted. The mean value and standard deviation of the numbers of the above mentioned cell organelles per unit of area of the cytoplasm (= 5,9 × 5,9 μm^2 = 100 points) were calculated In the same cells the presence of the following organelles was noted· dumb-bell shaped plastids, dumb-bell shaped mitochondria, large vesicles with a clear membrane, dilated cisternae of smooth ER (SER) and Golgi vesicles, rough ER (RER), SER, ribosomes, polysomes and microtubules. For each stage of development and each type of the above mentioned cell organelle the number of cells containing that type of organelle is given.

2.3 Statistical analysis

For each stage of development the mean value and standard deviation of the number of counted cell organelles per unit of area (100 points) in the sampled cell sections were calculated. The one way analysis of variance was applied to these values. If the result was significant at the 5% level, it was investigated with SCHEFFÉ's (1959) test for multiple comparison for each pair of mean values whether they are significantly different from each other It has to be remarked, however, that with Scheffe's test not such a pair can be distinguished, although the analysis of variance gives a significant result The same test was applied to the data concerning the area and greatest section of cells. Moreover, the correlation between the mean value of the area measured with EM and the section with LM for the same stage of development was tested with Spearman's correlation coefficient.

3 RESULTS

3 1. Morphological observations

3.1.1. The middle and late tetrad stage

The middle tetrad stage starts after formation of the callose wall between the four microspores When the footlayer or nexine I of the pollen wall becomes visible, the late tetrad stage commences.

During the middle tetrad stage the nucleus is situated near the exterior of the callose wall and a small strip of cytoplasm lies between the nucleus and the callose wall. The nucleus has a homogeneous nucleolus and the karyoplasm contains granules of approximately 30 nm (*fig. 1*) Sometimes the nuclear membrane shows invaginations, a phenomenon which has also been observed in the early

tetrad stage (WILLEMSE 1971b) In comparison with the preceding stages, some plastids have a small starch granule, while also dumb-bell shaped plastids are present The mitochondria contain an electron transparent material, including a few cristae. Also lipid granules are present, some of them connected with an electron transparent vesicle, forming a "lipid complex" Vesicles with a clear membrane and an electron transparent content are dispersed in the cytoplasm. Just before the start of the pollen wall formation cup-shaped Golgi bodies are observed (*fig 3*) Cisternae of SER, but few RER are present in the cytoplasm. Microtubules are absent Ribosomes as well as polysomes have been observed.

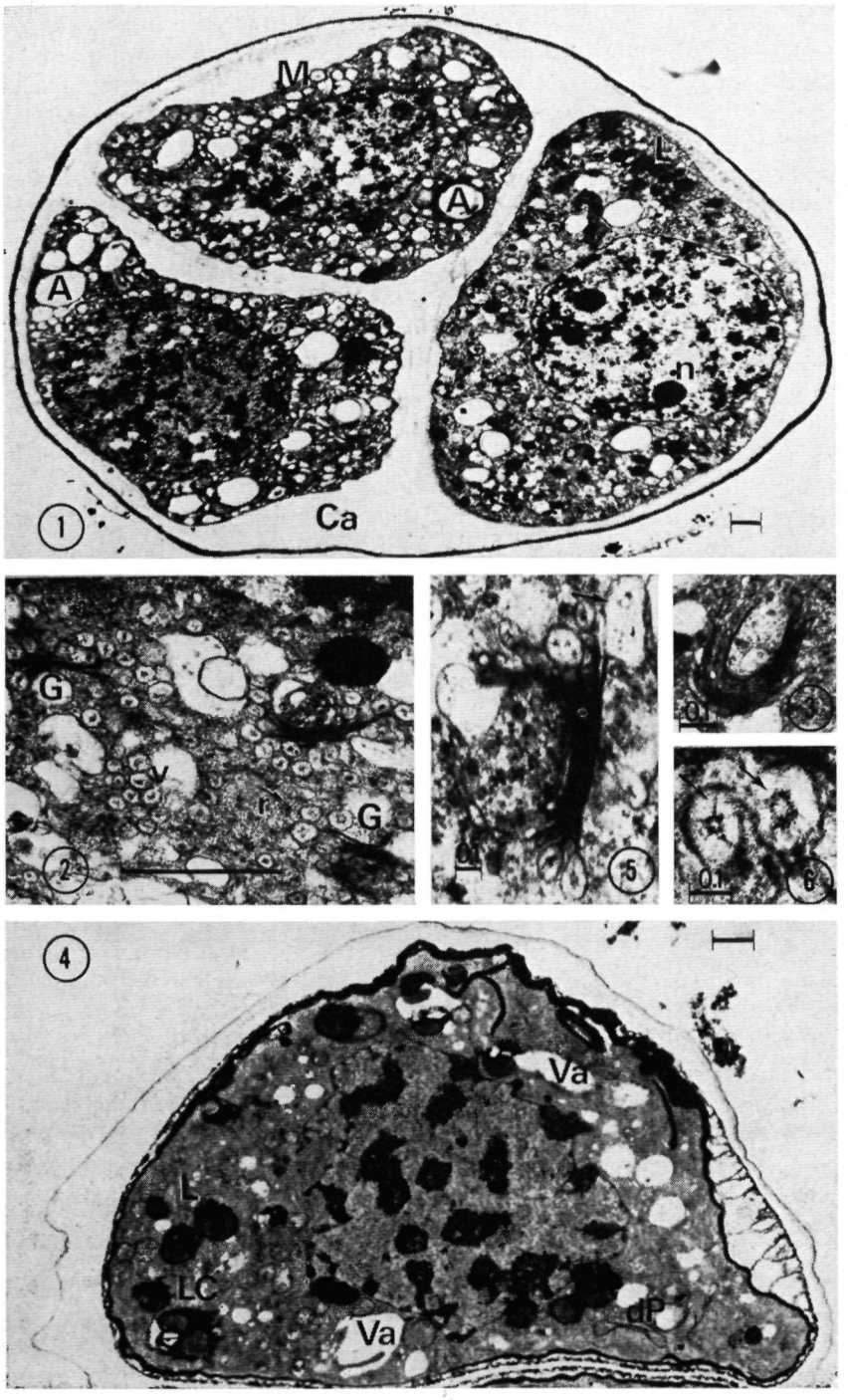
During the late tetrad stage the nucleus lies in the centre of the cell The chromatin of the nucleus is somewhat contracted (*fig 4*) The starch granules in the plastids have disappeared The number and size of the lipid granules increase, while many granules are surrounded by black dots. They are connected with electron transparent vesicles and form the "lipid complex". The vesicles with a clear membrane grow in volume and the vacuolisation of the cytoplasm starts (*fig 4*) The Golgi bodies disappear (*fig 20*). Strands of RER have been observed more frequently (*figs 13, 23*). The cytoplasm has many ribosomes; therefore, the polysomes are not clearly distinguishable, but they are present (*figs 20, 23*).

3.1.2 Pollen wall formation

Golgi bodies in the cytoplasm produce a large number of vesicles (*fig. 2*). The Golgi vesicles with a unit membrane have an electron transparent content. In their central part the electron dense granular and fine fibrillar material makes contact with the unit membrane by means of the fine fibrils (*figs 5, 6*) After lead citrate staining this inner structure becomes more distinguishable. The Golgi vesicles may fuse during their production on the Golgi body and during their transport (*figs. 5, 8*) During excretion of the Golgi material, the membrane of the vesicle makes contact and fuses with the plasma membrane (*figs 7, 8*). The content of the Golgi vesicles is excreted between the plasma membrane and the callose wall (*figs 8, 9*) At the point where Golgi material is excreted, the plasma membrane seems to be pushed away from the callose wall, due to the fusion with the membrane of the Golgi vesicles (*figs. 8, 9*) In this way, the plasma membrane becomes convoluted (*figs 11, 12*)

Just before excretion of the Golgi material, the plasma membrane is situated against the straight callose wall (*fig 10*) During excretion of the Golgi material, the contact between the plasma membrane and the callose wall persists locally (*figs 9, 11, 12*) Here the callose wall formation is not blocked and there a protrusion on the callose wall in the direction of the cell develops. The inner side of the callose wall loses its evenness and gets many protrusions on regular distances (*figs 11, 12, 17*)

Between the plasma membrane and the callose wall, the granular material originating from the Golgi vesicles changes gradually into a fine fibrillar network (*figs. 12, 14, 17, 18*) It precipitates against the callose wall initially between the protrusions and finally against the protrusions (*figs. 13, 17*). The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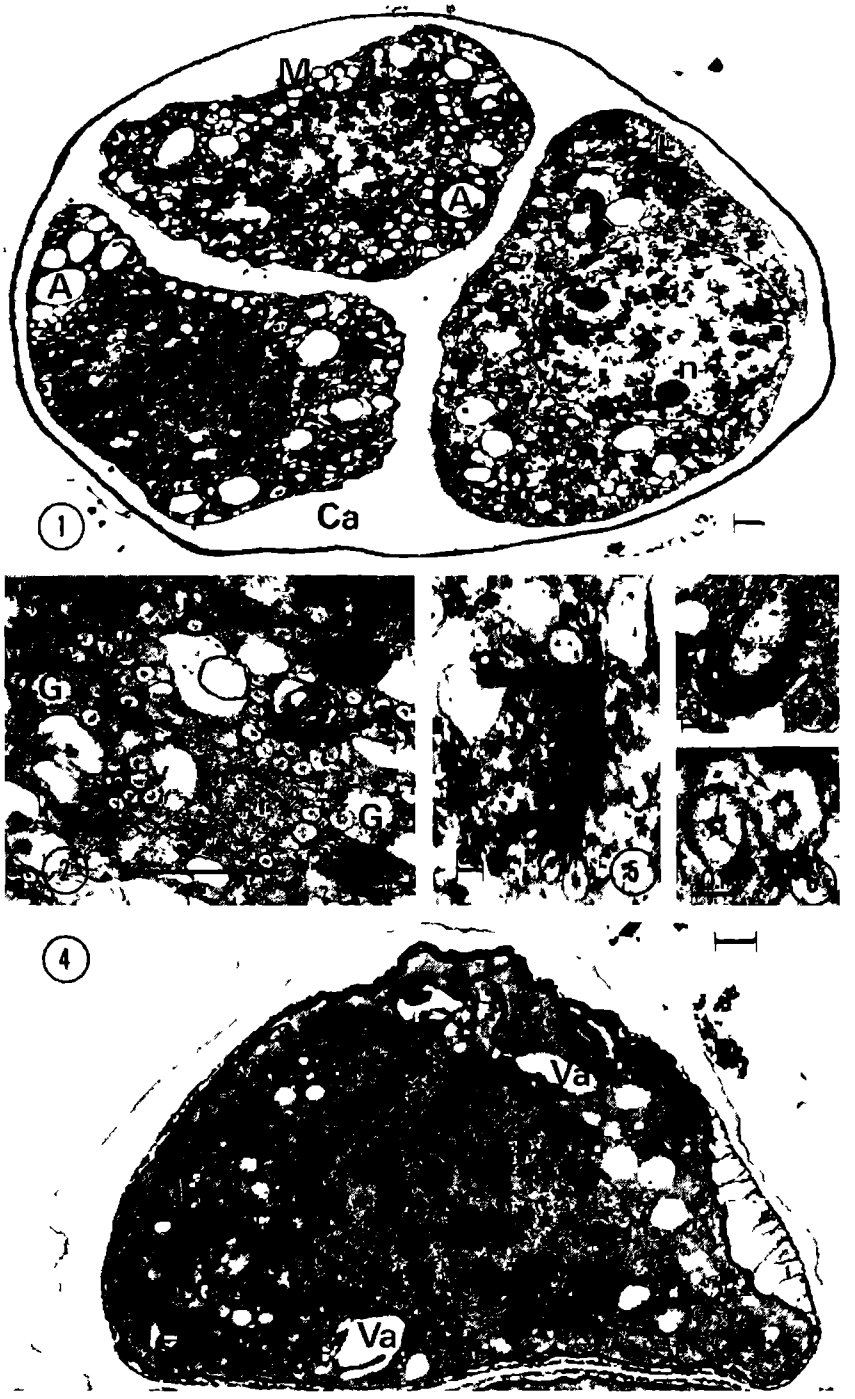
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material between the protrusions becomes more electron dense, marking the first sign of the tectum (*figs 13, 17*) Afterwards the bacula become visible as more electron dense structures Gradually very electron dense material becomes visible on tectum and bacula At the same time this material appears also locally along the plasma membrane, as the footlayer or nexine I (*figs 14, 18*)

This process can be followed easily on those places where the sacci are formed The protrusions on the callose wall and connected with the plasma membrane are branched and long with numerous contents of Golgi vesicles in between (*figs 12, 15, 17*) The material of the Golgi vesicles becomes more fibrillar starting between the protrusions and precipitates against the callose wall between the protrusions Above the plasma membrane the Golgi material remains a longer time more granular (*fig 17*) In a tangential section the protrusions are round and situated at somewhat regular distance (*figs 15, 16*) Finally, all the granular material from the Golgi vesicles changes into a fine fibrillar network (*fig 18*) The fine fibrillar material of the Golgi vesicles precipitates and condenses against the callose wall and its protrusions (*fig 18*) In these joint fibrils more electron dense material appears on the places of tectum, bacula and along the plasma membrane The now completely fibrillar Golgi material makes contact with the footlayer, bacula and tectum (*fig 18*)

The Golgi bodies now disappear in the cytoplasm and no contact exists between the plasma membrane and the callose wall (*figs 14, 18*) No tectum and bacula are present in the region between the sacci, which is the future germination pole of the pollen tube Under the plasma membrane a sheet of ER has not been observed here

The very electron dense material, probably the sporopollenin, still increases in size in the sexine and nexine I A number of long tapes is visible along the plasma membrane and on the boundary extending in the cytoplasm (*figs 4, 19, 20*) In these electron dense tapes, lamellae of unit membrane dimension are present These lamellae have not been observed in the tectum and bacula (*figs 22, 28*) The plasma membrane follows the tapes deep into the cytoplasm (*fig 21*) In the cytoplasm the tapes not always border on unit membranes (*fig 24*) ER is sometimes found parallel to the tapes (*fig 23*) Also a close contact with lipid granules or the "lipid complex" exists with the tapes (*figs 20, 24*) In the cytoplasm centres of circular bowed membranes have been observed

Fig 1 M Tetrad stage the nucleus with homogeneous nucleolus (n) is situated near the exterior of the callose wall (Ca) In the cytoplasm are visible plastids with a starch granule (A), mitochondria (M) and lipid granules (L), × 4,600

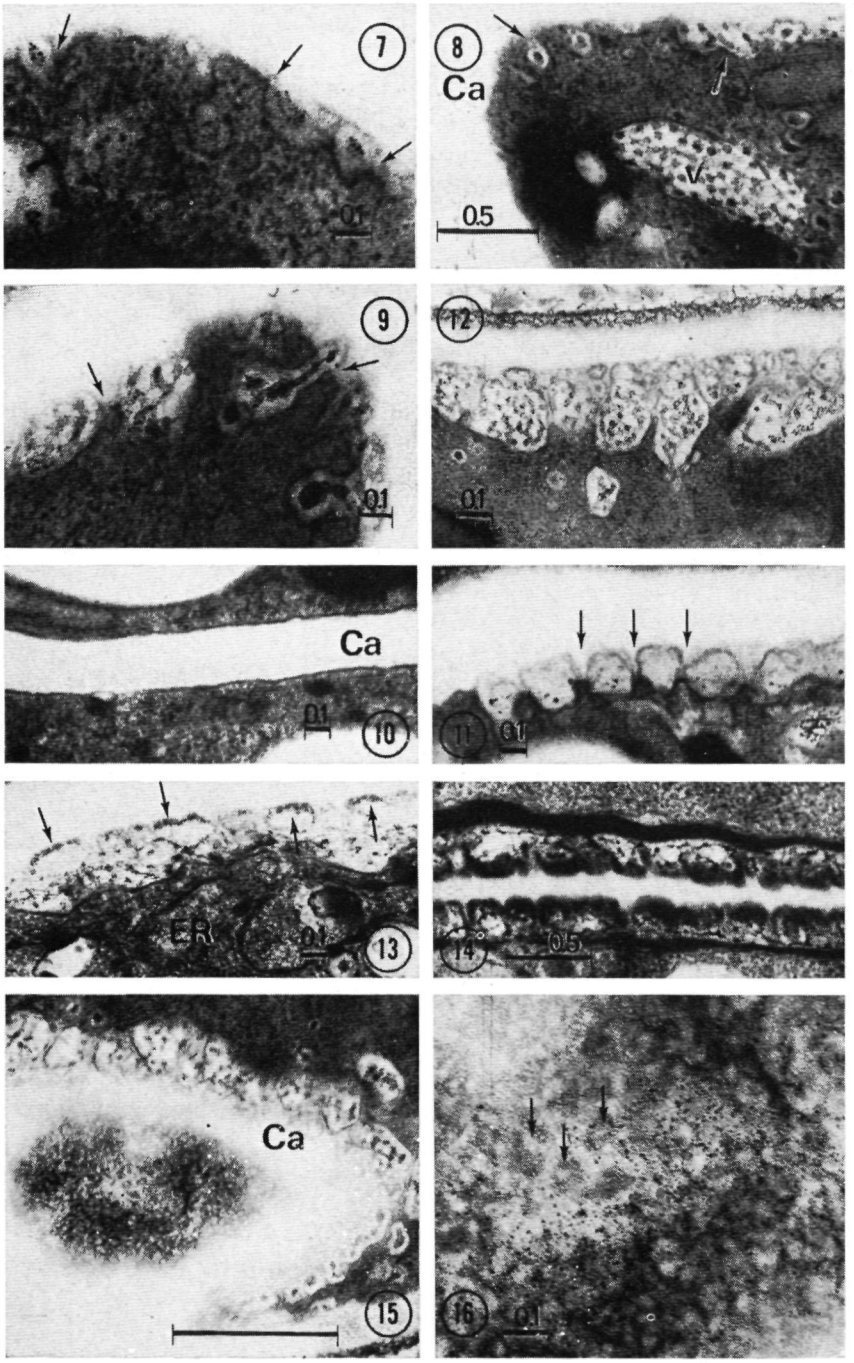
Fig 2 Detail cytoplasm with Golgi bodies (G), Golgi vesicles (v) and ribosomes (r), × 24,000

Fig 3 Cup-shaped Golgi body, × 54,000

Fig 4 Late tetrad stage in the cytoplasm large lipid granules (L) and the "lipid complex" (LC) and a dumb-bell shaped plastid (dP) Vacuoles (Va) appear, × 6,200

Fig 5 Detail of Golgi body with a fused Golgi vesicle (arrow), × 37,000

Fig 6 Detail of granular and fine fibrillar content (arrow) of the Golgi vesicle, × 69,000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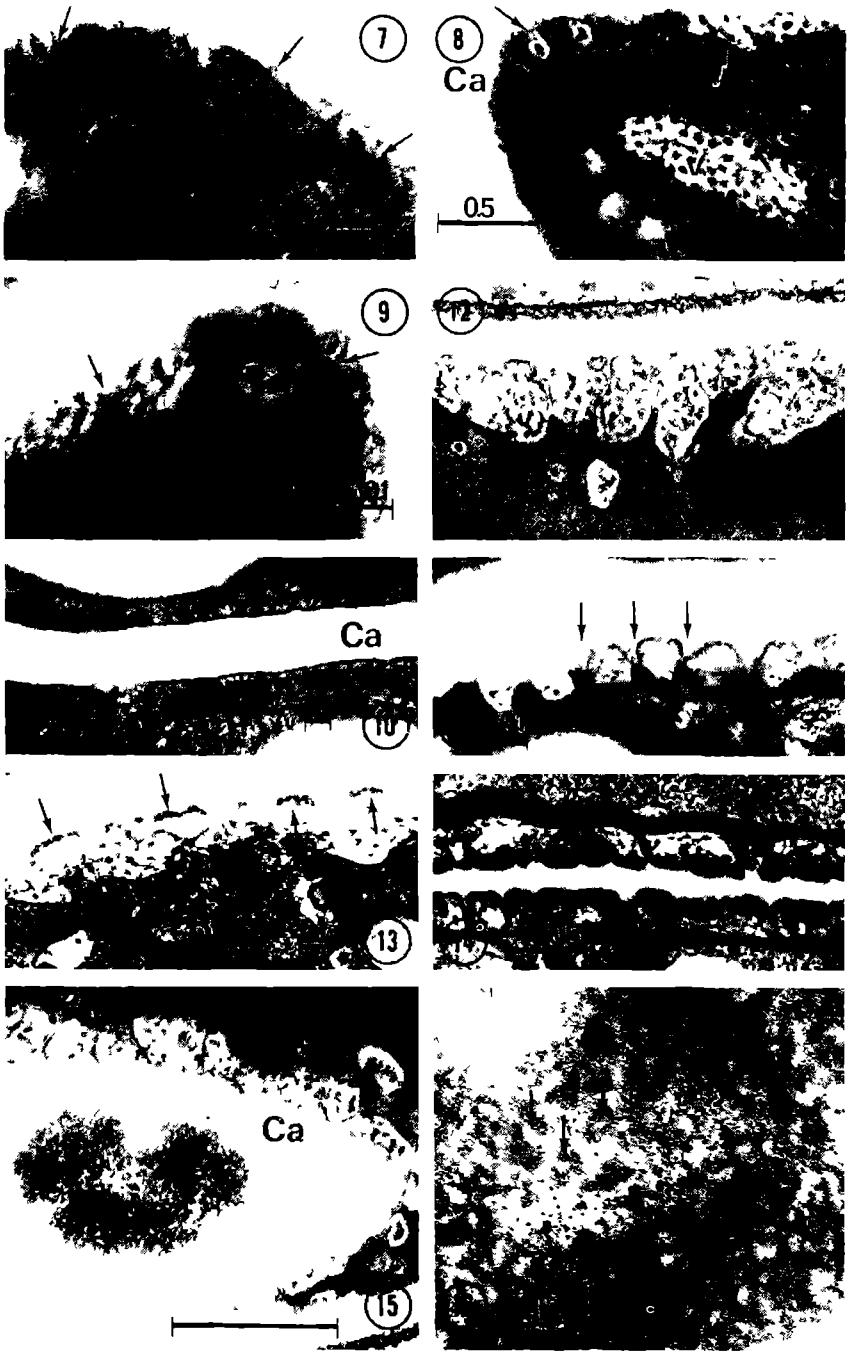
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(*figs 21, 22*) Between and on their membranes the very electron dense material is present Dark granular material is found in the bowed membranes (*figs 25, 26*) The pollen wall sexine and nexine I are well developed before the microspores are set free from the callose wall (*fig 29*)

3 1 3 The young microspore

Starting at the sacci, the callose wall and possibly the thin cell wall are partly broken down by external enzymatical degradation (*fig 18*) In the liberated microspore the nucleus moves to the future germination pole and subsequently to the cell centre In the cytoplasm many vacuoles appear and some plastids contain a starch granule No further changes of cell organelles have been observed (*fig 27*)

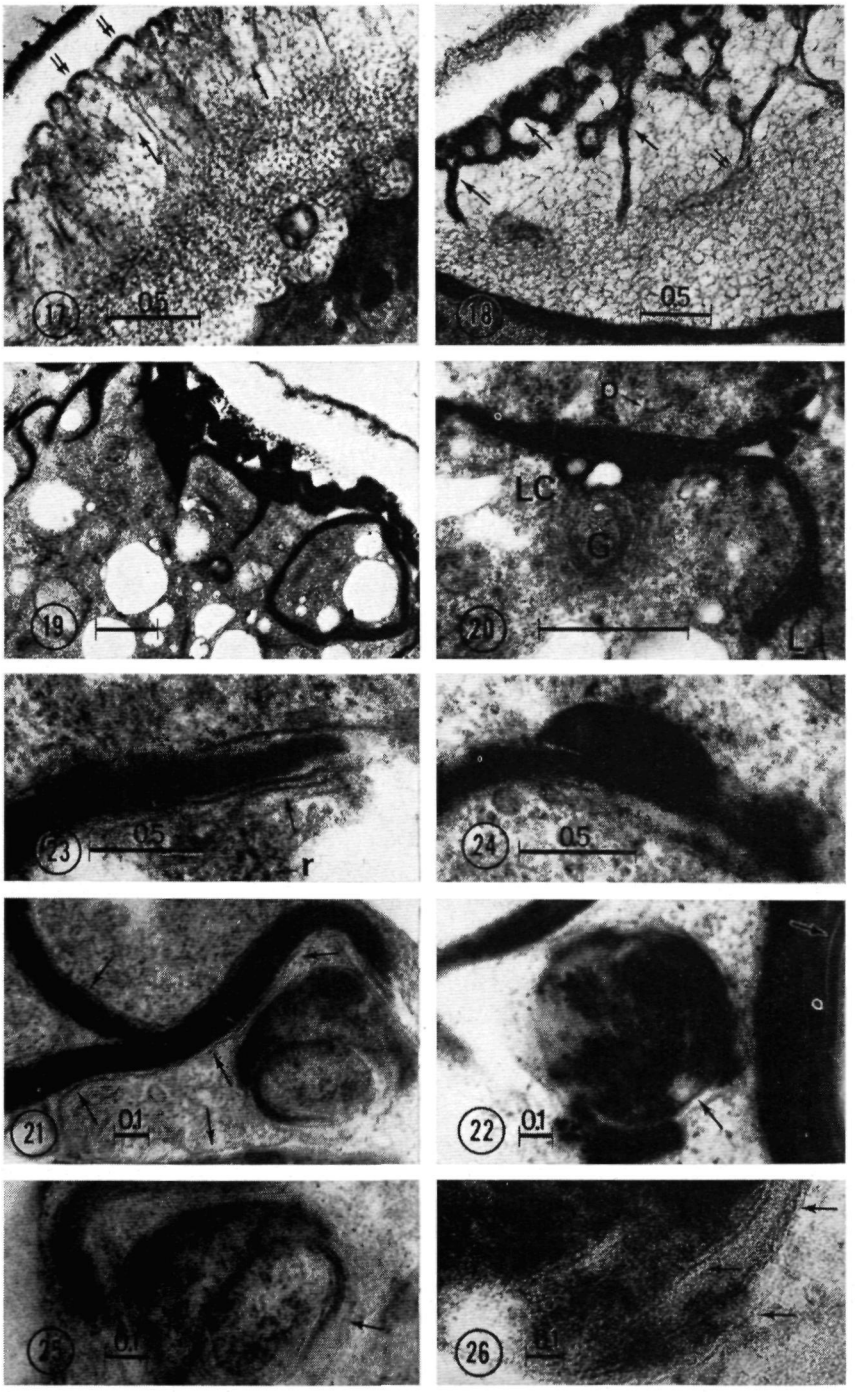
In the young microspore the tectum and bacula swell The nexine I grows and the layers of the nexine II appear (*figs 30, 31*) In the completely developed pollen wall the nexine II is not distinguishable from the nexine I (*fig 32*) After the start of the formation of the intine the pollen wall formation stops, including the thickening of the tectum and bacula The nexine is thicker on the germination side than on the opposite side (*fig 27*)

The content of the sacci changes during the growth of the sacci In comparison with a later stage of development, in freeze-etched pictures a cristallization pattern in the content of the sacci different from water has been found (*figs 33, 34*)

3 2 Quantitative approach

Diagram I shows the changes of the largest cell section, the cell section area and the number of some cell organelles during the different stages of microsporogenesis up to the young microspore stage The results are given of the determinations of the cell and cytoplasm area, obtained from EM photographs of cell sections and from LM photographs of intact cells The mean values with standard deviation of the number of some cell organelles are noted per unit of area of the

Fig 7 Excretion of the Golgi material membrane of Golgi vesicle fuses with the plasma membrane (arrow), × 49,000
Fig 8 Fused Golgi vesicles in the cytoplasm (v), content of the Golgi vesicles is outside the cell (arrow), × 29,500
Fig 9 Plasma membrane shows undulations by its fusion with the membrane of the Golgi vesicle (arrow), × 46,000
Fig 10 Plasma membrane situated against the straight callose wall (Ca), × 36,500
Fig 11 Protrusions on the callose wall, note the contact with the plasma membrane (arrow), × 42,000
Fig 12 Formation of the callosic protrusions on the plasma membrane at the place of the future saccus, × 52,000
Fig 13 Between the protrusions the precipitation of electron dense material starts (arrow) Note the ER in the cytoplasm, × 35,000
Fig 14 Appearance of the footlayer along the plasma membrane, × 25,000
Fig 15 Local tangential section of the callose wall (Ca), × 24,500
Fig 16 Detail Fig 15, arrows arrangement of the round protrusions, × 73,000.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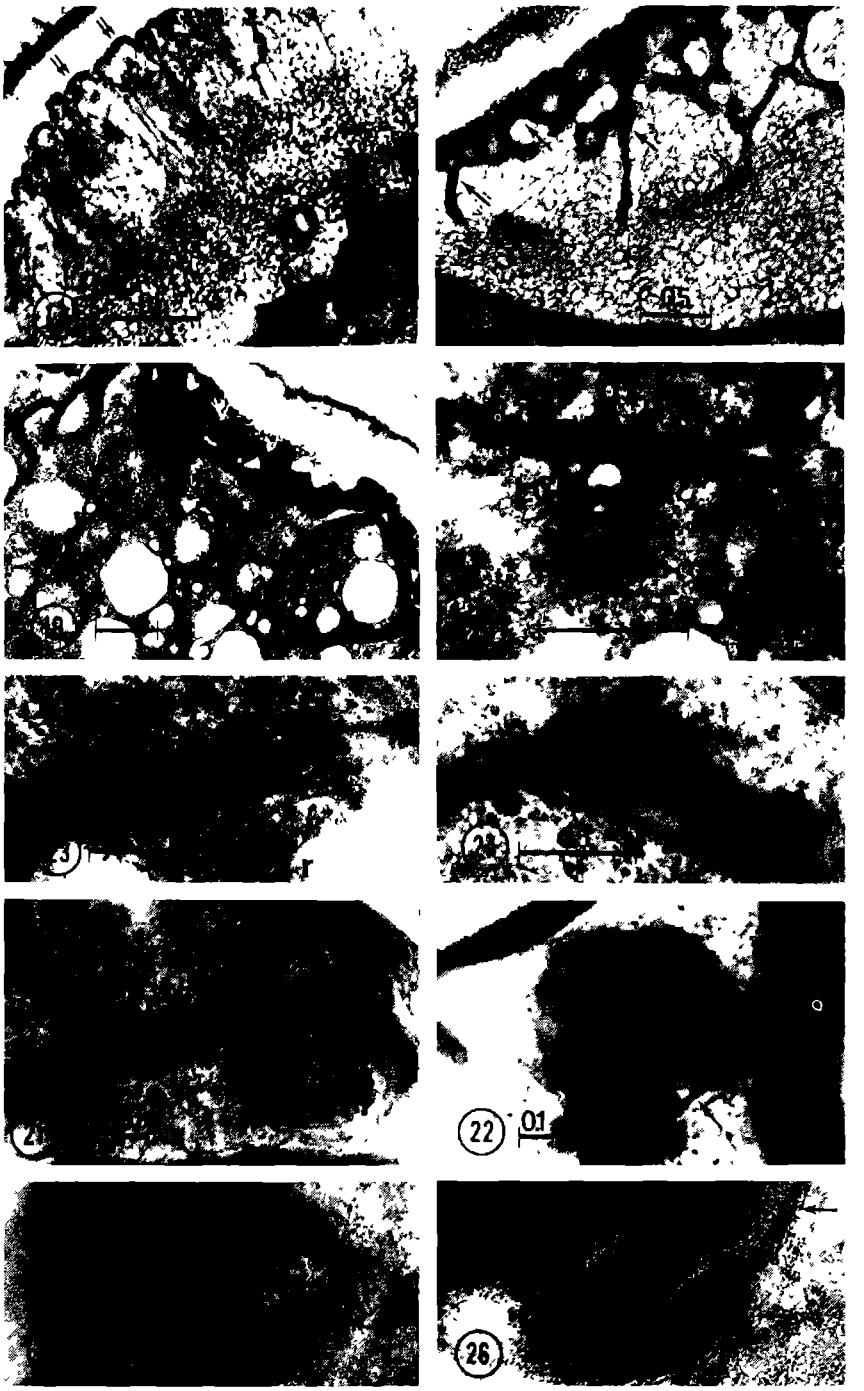
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Table 1 The portion of cell sections in which the indicated cell organelles are present during the subsequent stages of microsporogenesis

	Stages										
	EZ	LZ	P	D	DA	MI	I	MII	ET	MT	LT
Total number of investigated cell sections	5	5	5	7	5	7	5	6	5	5	5
Dumb-bell shaped plastids	8	6	6	7	6	4	4	1	8	10	6
Dumb-bell shaped mitochondria	2	6	2	1	0	0	2	1	0	0	2
Large vesicles	10	10	10	10	10	10	10	10	10	10	10
RER	10	8	10	1	0	0	0	0	0	2	4
SER	8	10	10	10	10	10	10	10	10	10	4
Ribosomes	10	10	10	10	10	10	10	10	10	10	10
Polysomes	2	10	10	10	10	2	0	8	10	2	4
Microtubules	2	6	8	10	10	10	6	10	2	0	2

cytoplasm (= 100 points) In the diagram the results of the Scheffe's test are also included

Table I gives the numbers of cell sections in which some organelles are present during the different stages of development

The results of the statistical analysis can be summarized as follows·

1. All measurements in the different stages by means of the one way analysis of variance for the numbers of cell organelles and for the cell area and largest section are significant at the 5% level
2. Scheffé's test gives significant results for the plastids with a starch granule, plastids, Golgi bodies and the measurement of the largest section of the cell on LM photographs, as indicated in diagram I

Fig 17 Formation of the saccus Long protrusions with fine fibrillar material (arrow) in between, against the plasma membrane a zone of more granular material Electron dense material appears against the callose wall (arrows), × 27,000

Fig 18 Formation of the saccus Against the callose wall and its protrusions electron dense material precipitates (arrow) All Golgi material is a fine fibrillar network now Note the condensation of this material on the base of the protrusion (arrows) and the connection with the footlayer, × 20,000

Fig 19 Late tetrad stage along the plasma membrane and in the cytoplasm electron dense tapes, × 9,200

Fig 20 Electron dense tape with lipid granule (L) and "lipid complex (LC) Note the disappearing Golgi body (G) and polysomes (p), × 21,900

Fig 21 Detail electron dense tape in the cytoplasm Note the plasma membrane along this tape (arrow), × 45,000

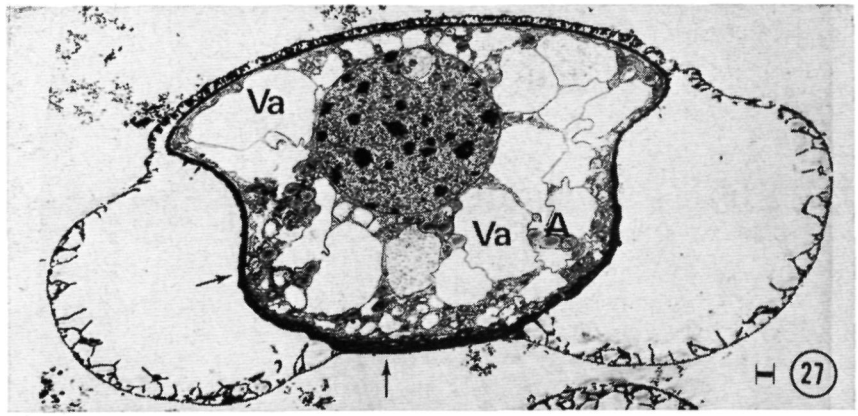
Fig 22 Lamellae of unit membrane dimension in the electron dense tapes (arrow) Survey centre of circular membranes with electron dense material, × 43,000

Fig 23 ER along the electron dense tape (arrow), ribosomes (r) in the cytoplasm, × 34,500

Fig 24 Detail fig 20, electron dense tape with lipid granule, × 34,500

Fig 25 Detail fig 21, circular thin membranes (arrow), × 30,000

Fig 26 Detail fig 22, membranes on which electron dense material appears (arrow), × 59,000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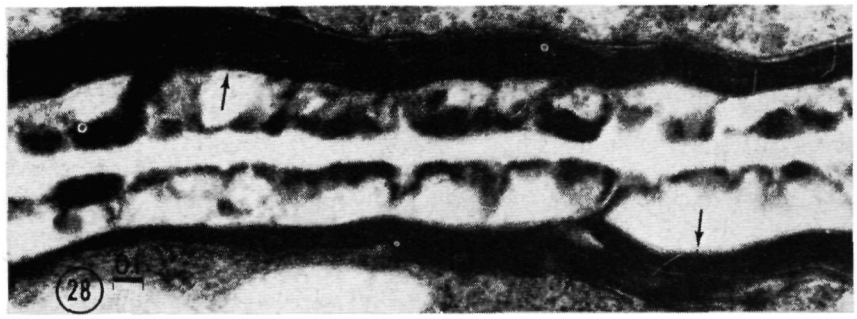
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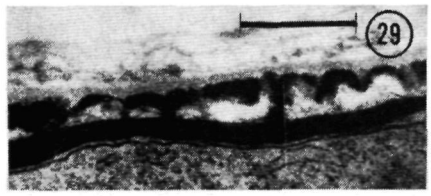
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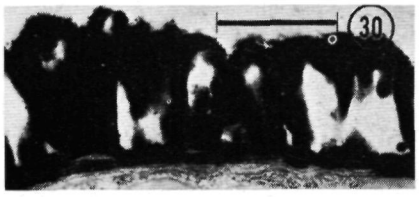
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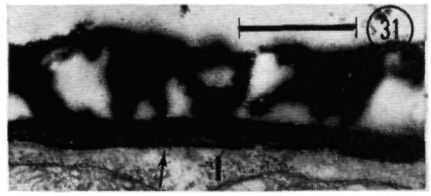
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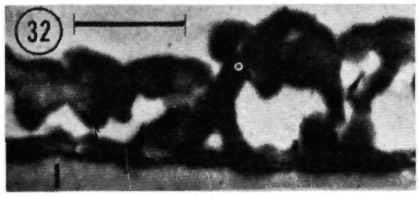
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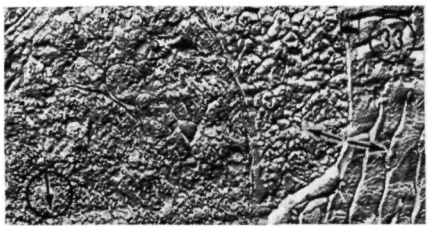
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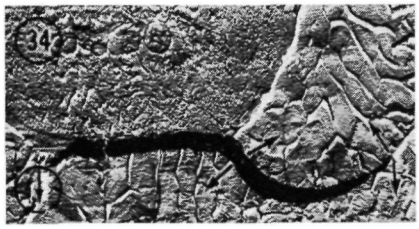
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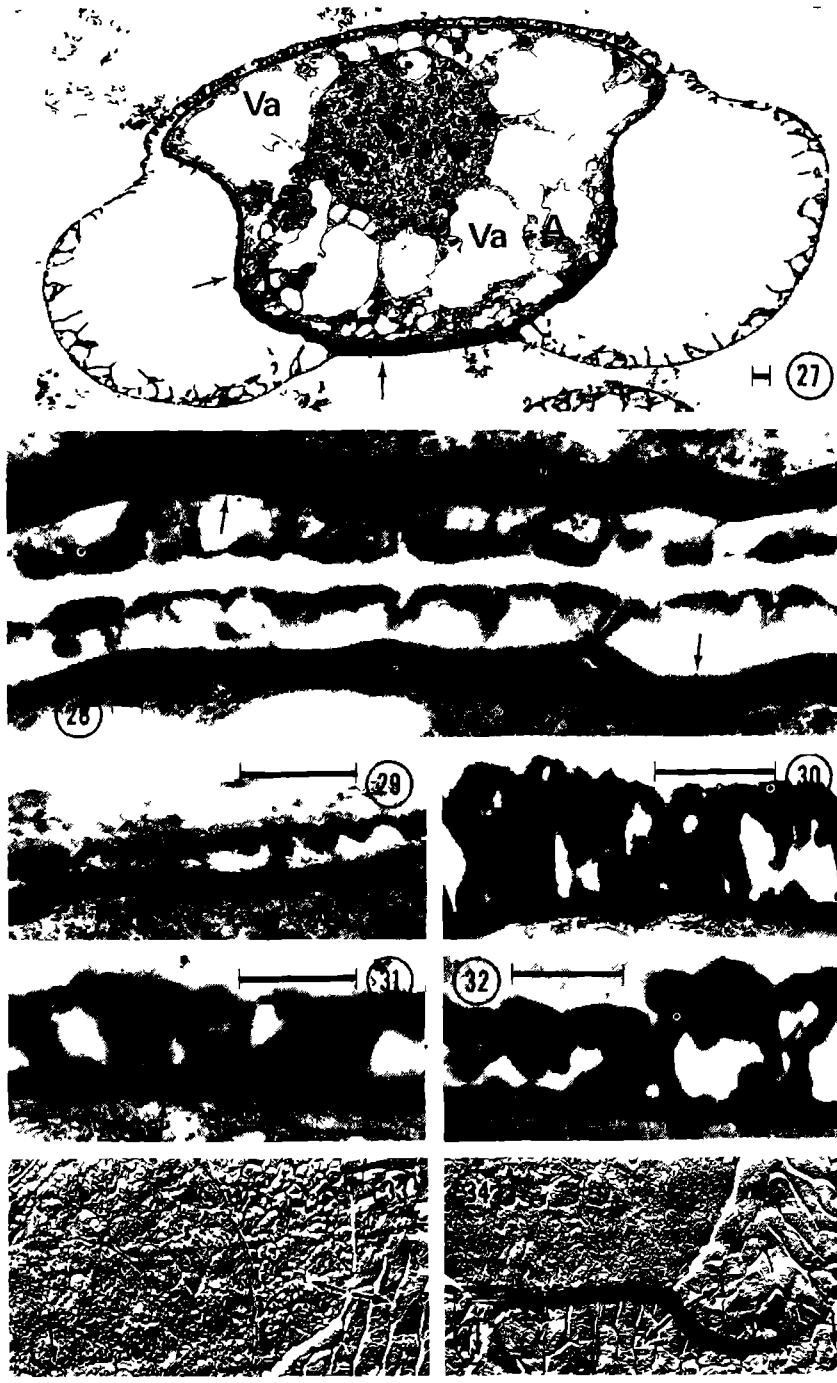
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3 A significant correlation exists between the mean values per stage of the cell section area measured on EM photographs and those of the largest section of the cell measured on the LM photographs (Spearman's coefficient was 0 83, $p < 0{,}005$)

4 DISCUSSION AND CONCLUSION

4 1 Pollen wall formation the formation of the sexine

In the pollen wall formation of *Pinus sylvestris* the template of the exine pattern is a result of excretion of Golgi material and a continued formation of callose on the plasma membrane between the excreted Golgi material The more granular fine fibrillar Golgi vesicle content changes after excretion into a fibrillar network, which precipitates and condenses against the callose wall at first between the callosic protrusions and subsequently against the protrusions The callose wall functions as a template This is in accordance with the opinion of WATERKEYN & BIENFAIT (1970)

The Golgi vesicles contain the primesexine, the basic material for the tectum and bacula This material consists of polysaccharides (LEPOUSE 1970) and may be hemicellulose and/or pectic-like (DICKINSON & BELL 1970) It seems to be sensitive to a poststaining with leadcitrate From the response to uranyl acetate and leadcitrate HESLOP-HARRISON (1971a) suggests that it may contain a lipoprotein However, the nature of the electron transparent content of the Golgi vesicle remains unknown As soon as the content of a Golgi vesicle has been excreted, it causes a break of the contact between plasma membrane and callose wall The plasma membrane is locally replaced by fusion with the membrane of the Golgi vesicle The plasma membrane does not retract The undulations of the plasma membrane are caused by the excretion of Golgi material as has been suggested by ECHLIN & GODWIN (1968) They are no artifacts as HESLOP-HARRISON (1971a) suggested In the present author's opinion,

Fig 27 Young microspore, cytoplasm has vacuoles (Va) and plastids (A) with a starch granule Note the thickness of the nexine (arrow), × 2,300

Fig 28 Late tetrad stage lamellae of unit membrane dimensions are present only in the nexine I (arrow), × 46,000

Fig 29 Pollen wall of a microspore just breaking out, tectum and bacula are small, × 17,200

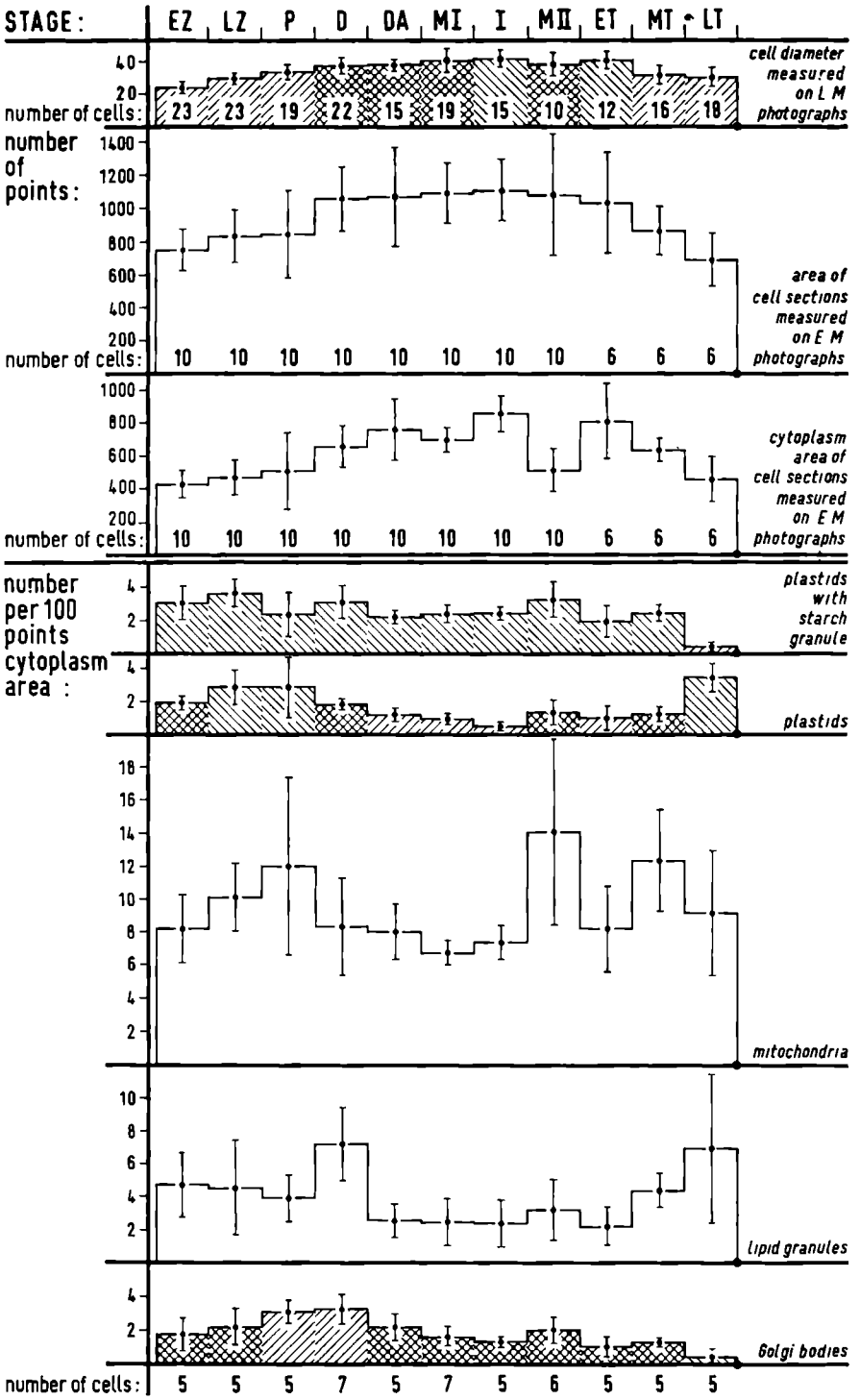
Fig 30 Tectum and bacula are thickening in a young microspore, the formation of the nexine II starts, × 18,200

Fig 31 The nexine II (arrow), thickening of the tectum and bacula stops, the intine formation starts (I), × 18,000

Fig 32 Pollen wall of a ripe pollen grain nexine I and II are both one layer, size of the tectum and bacula corresponds with those of fig 30 and 31, × 17,300

Fig 33 Freeze-etched microspore before the growth of the sacci Note the crystallization pattern in the saccus and the surrounding water (arrow), × 1,980

Fig 34 Young microspore the sacci have been developed, the content of the saccus is the same as the surrounding water (arrow), × 1,980
Unless mentioned otherwise, the line on the figures represents a length of 1 μm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number of points:
number of cells:
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area of cell sections measured on E M photographs
cytoplasm area of cell sections measured on E M photographs
number per 100 points cytoplasm area :
plastids with starch granule
plastids
mitochondria
lipid granules
Golgi bodies
number of cells:
5 5 5 7 5 7 5 6 5 5 5

it seems not reasonable to support DICKINSON's (1970) view that a considerable pressure may exist in the space between plasma membrane and callose wall

The localization of the protrusions is already determined during the very first excretion of the Golgi material The Golgi vesicles are not directed by microtubuli during their transport to the plasma membrane In the first instance they are juxtaposed outside the plasma membrane Between the excreted Golgi material the contact between plasma membrane and callose wall persists, here the origin of a protrusion may be found The contact between plasma membrane and callose wall seems necessary for the formation of the callosic protrusions

The excretion of Golgi material probably represents a rapid process (ECHLIN & GODWIN 1968, BROWN 1969) In pine the excretion of Golgi material occurs simultaneously with the formation of the protrusions on the callose wall This does not correspond with the theory that the whole onus of pattern establishment could be placed upon the plasmalemma and that it is associated with cytoplasmic structures including ER (HESLOP-HARRISON & DICKINSON 1969, HESLOP-HARRISON 1971a) The observations in pine do not corroborate the hypothesis of DICKINSON (1970) that after formation of the primexine the origin of the plasma membrane protrusions should be connected with Golgi vesicle excretion, which is directed by microtubules

4 2 The formation of the nexine

In pine the sporopollenin is produced on membranes of the microspore During the last stages of primesexine formation electron dense material appears first at the future place of the tectum, then at the bacula and finally along the plasma membrane An opposite sequence is more generally found first the appearance of the probacula and thereafter of the tectum (SKVARLA & LARSON 1966, ECHLIN & GODWIN 1968, DICKINSON 1970) In *Pinus sylvestris* the sporopollenin of the nexine I and II is produced on the plasma membrane or on membrane structures in the cytoplasm However, no lamellae of unit membrane dimension in the material of the sexine have been observed This is contrary to the suggestion of HESLOP-HARRISON & DICKINSON (1969) that in all elements of the exine the sporopollenin always is deposited on lamellae of unit membrane

Diagram 1 The mean values and standard deviations of the cell section area and the largest section of the cell (= cell "diameter') are given in numbers of point per stage of development

The mean values and standard deviations of the number of organelles per 100 points cytoplasm area are given for each stage of development The numbers of investigated cell sections or cells ("number of cells on the diagram) are stated on the basis of the diagram

The results of the Scheffe s test are indicated by means of an orientated shading Double shading indicates that the corresponding mean values do not differ significantly from any other mean value in the same diagram If two or more columns in the diagram have the same type of shading in one direction the corresponding mean values do not differ significantly

dimension The electron dense material of the sexine, probably sporopollenin, appears before the formation of the nexine I It is transported from the microspore cytoplasm to the tectum and bacula, or along the fibrillar network of the Golgi material (*figs 14, 18*), or as an electron transparent precursor After formation of the footlayer, the transport of electron dense material to the elements of the sexine may be blocked But after the breakdown of the callose wall the tectum and bacula are still thickening Sporopollenin produced by the tapetal cells is possibly absorbed now in the sexine As has been described elsewhere (WILLEMSE 1971), the tapetal cells produce globules on which sporopollenin is present In that case an electron transparent precursor of sporopollenin may exist in the tapetal fluid Another possibility would be that the transport of electron dense material to the sexine is not blocked by the nexine I In that case all sporopollenin for the pollen wall may originate from the microspore

The long tapes of nexine I are mainly observed outside the plasma membrane, but in pine some tapes seem to be situated partly or completely in the cytoplasm The bundles of unit membranes observed in the cytoplasm on which electron dense material appears, may possibly be considered as the origin of the tapes consisting of sporopollenin These unit membranes are connected with electron dense granular material, possibly originating from the lipid granule A connection of lipid granules or the "lipid complex" with the tapes has also been observed A relation may exist between the lipid granules, which are very voluminous in this stage, and sporopollenin production, as may occur in *Eulophidium* (CHARDARD 1971) and *Asclepias* (LINSKENS & SUREN 1969) The deposition of sporopollenin on membranes occurs in *Pinus sylvestris* inside as well as outside the cytoplasm The high number of ribosomes in the cytoplasm and the decrease of starch in the plastids may also be related to the pollen wall formation

A difference exists in the composition of the sexine and the nexine The sexine, partly derived from material of the Golgi vesicles, contains more polysaccharides than the nexine The callose of the protrusions is broken down during the enzymatical removal of the callose wall

In pine the pollen wall formation may be analogous to the callose wall formation (WILLEMSE 1971b) Golgi material forms after excretion a network of fine fibrillar material, probably consisting of hemicellulose and/or pectic substances, which becomes impregnated by callose or sporopollenin These wall substances originate from and are orientated by means of membranes, mainly outside the microspore

4 3 Formation of the exine pattern and the swelling of the sacci

The formation of the exine pattern in pine depends on the quantity of excreted Golgi material The first steps of Golgi material excretion are very important for the localization of the protrusions on the callose wall In the area between the sacci the nucleus lies near to the callose wall Here the small strip of cytoplasm contains few Golgi vesicles, protrusion formation lacks and no sexine is formed The formation of the sacci is related to a high number of Golgi vesicles The nexine is formed and orientated by membranes, mainly the plasma mem-

brane. During the pattern formation the number of Golgi vesicles, the content of which locally has been excreted, corresponds to the local quantity of Golgi vesicles in the cytoplasm and the localization of the nucleus. An analogous situation during cell wall formation in *Pleurochrysis scherffelii* has been reported by BROWN (1969).

In *Pinus banksiana* the content of the sacci gives a PAS+reaction (DICKINSON & BELL 1970) In *Pinus sylvestris* the network of fine fibrills in the sacci disappears. During a short time the sacci contain material, which is chemically different from water The cytoplasm and the sacci of the microspore probably swell osmotically by uptake of water.

The reappearance of starch in the plastids and the vacuolisation of the cytoplasm of the microspore may be a consequence of an uptake of water and possibly of sugars from the tapetal fluid No imbibition occurs as may be concluded from the simultaneous swelling of the whole microspore, the disappearance of the fine fibrillar network in the sacci and the watery content of the just swollen sacci.

4 4. Quantitative approach

The number of some types of cell organelles are determined per unit of area of the cytoplasm This density of organelles of each stage of development can be compared in relation to each other and a quantitative survey of a part of the microsporogenesis may be given A determination of the absolute number of some types of cell organelles demands a complete series of subsequent cell sections This is not practicable at a high number of different stages of development

The one way analysis of variance gives significant results in all series This means that during microsporogenesis significant changes in cell volume and in number of some types of cell organelles occur. However, the measured mean values and standard deviations of the number of cell organelles are very fluctuating A clear regular or systematic increase or decrease of numbers of organelles could not be stated.

These fluctuations may be due to the different reaction of the cell on the same preparation treatment, dependent on the stage of development, or to an influence of micro- and macro-climatological conditions. Of more influence is the low number of investigated cells and the possible polarity of some cell organelles in the cell, which cannot be excluded. Therefore, the results of this quantitative approach are not completely reliable.

One regularity in the results may be remarked: the density of cell organelles decreases or increases when the volume of the cell changes From diakinesis till the early tetrad stage an increase of cell volume and cytoplasm volume and a decrease of the density of cell organelles takes place During the second division stage the volume of the cytoplasm decreases and the density of cell organelles increases (see diagram I) This is an indication that no increase or decrease of the cell organelles may be expected This is in agreement with the observation that chondriosomes, sphaerosomes and proplastids do not change in number during the meiosis in microsporogenesis of *Impatiens glandulifera* (STEFFEN & LANDMANN 1958).

On the basis of the quantitative results it is not reasonable to make a difference between the early zygotene and late zygotene Differences in the number of lipid granules, plastids and Golgi bodies are demonstrated between the early tetrad, middle tetrad and late tetrad stage This subdivision of the tetrad stage seems reasonable

4 5 The cell size

The size of the cell increases from zygotene till interphase II and decreases subsequently after the early tetrad stage to approximately the same size as in zygotene This is shown both by the mean values of the area and, according to Scheffe's test, of the largest section of the cells in the different stages of development and is affirmed as a positive correlation between the size and the largest section It may be concluded that the cell volume changes during microsporogenesis The increase of volume is not only the result of the enlargement of the nucleus and of the nuclear region during the cell divisions, but also of the increase of the cytoplasm area as is shown in diagram 1 Only during the second division stage the area of the cytoplasm is compressed

A relation to the changes in the spindle may exist, because the increase of volume occurs during the division stages (BARLOW 1970) But from prometaphase till telophase, the number of Golgi vesicles and dilated SER in the cytoplasm increases also An osmotical change in relation to the contraction of the chromosomes may not be excluded as a cause of an increase of volume

4 6 The cell organelles

The mean values of the number of mitochondria show a considerable fluctuation during all stages of development In the series no differences could be given by the Scheffe's test Therefore, it is impossible to conclude here whether the observed dumb-bell shaped mitochondria are dividing mitochondria

The number of lipid granules increases during the diplotene and the late tetrad stage, being stages of wall formation

The fluctuation in the number of mitochondria and lipid granules is partly due to the changes in the volume of the cytoplasm

The number of Golgi bodies is large during the pachytene and diplotene stages of callose wall formation From diakinesis till the late tetrad stage the number of Golgi bodies decreases, during the late tetrad stage the number is very low, the Golgi bodies disappear It may be possible, that in the different cell processes not the same population of Golgi bodies functions During cell plate formation a polarity of Golgi bodies cannot be excluded

Till the late tetrad stage the number of plastids with a starch granule shows less differences During this stage the starch in the plastids disappears

The mean values of the number of plastids show a large fluctuation during all stages of development During the late zygotene and the diplotene the mean value of the number of plastids and also their standard deviation are large, the reason of these high numbers is not clear The number of plastids increases during the late tetrad stage according to Scheffé's test This is a result of dis-

appearance of the starch in the plastids The sum of the mean values of the plastids with a starch granule and the plastids of each stage of development shows that during microsporogenesis from the zygotene till the young microspore stage the fluctuation decreases It can be concluded that the whole population of plastids in the cell shows no differences in number during this part of the microsporogenesis

Although dumb-bell shaped plastids are present in all stages, also during the division stages in which plastids are not supposed to divide (MICHAELIS 1962, ANTON-LAMPRECHT 1967), it is difficult to conclude whether they are dividing plastids A degeneration of the plastids has not been observed

Vesicles with a clear membrane, SER and ribosomes are present in all stages The presence of microtubules is related to the division stages RER is absent during the division stages and changes during diplotene in SER RER has been observed again in middle tetrad stage Polysomes are present before and during the callose wall formation and disappear during diakinesis A new population is observed in interphase II and possibly also in telophase II

4 7 Survey of the quantitative approach

As mentioned above, the analysis of variance gives a significant result, but no clear systematical increase or decrease of the density of cell organelles could be found In the description of the counted cell organelles the increase or decrease in a distinct stage could be stated, in particular in relation to different cell processes

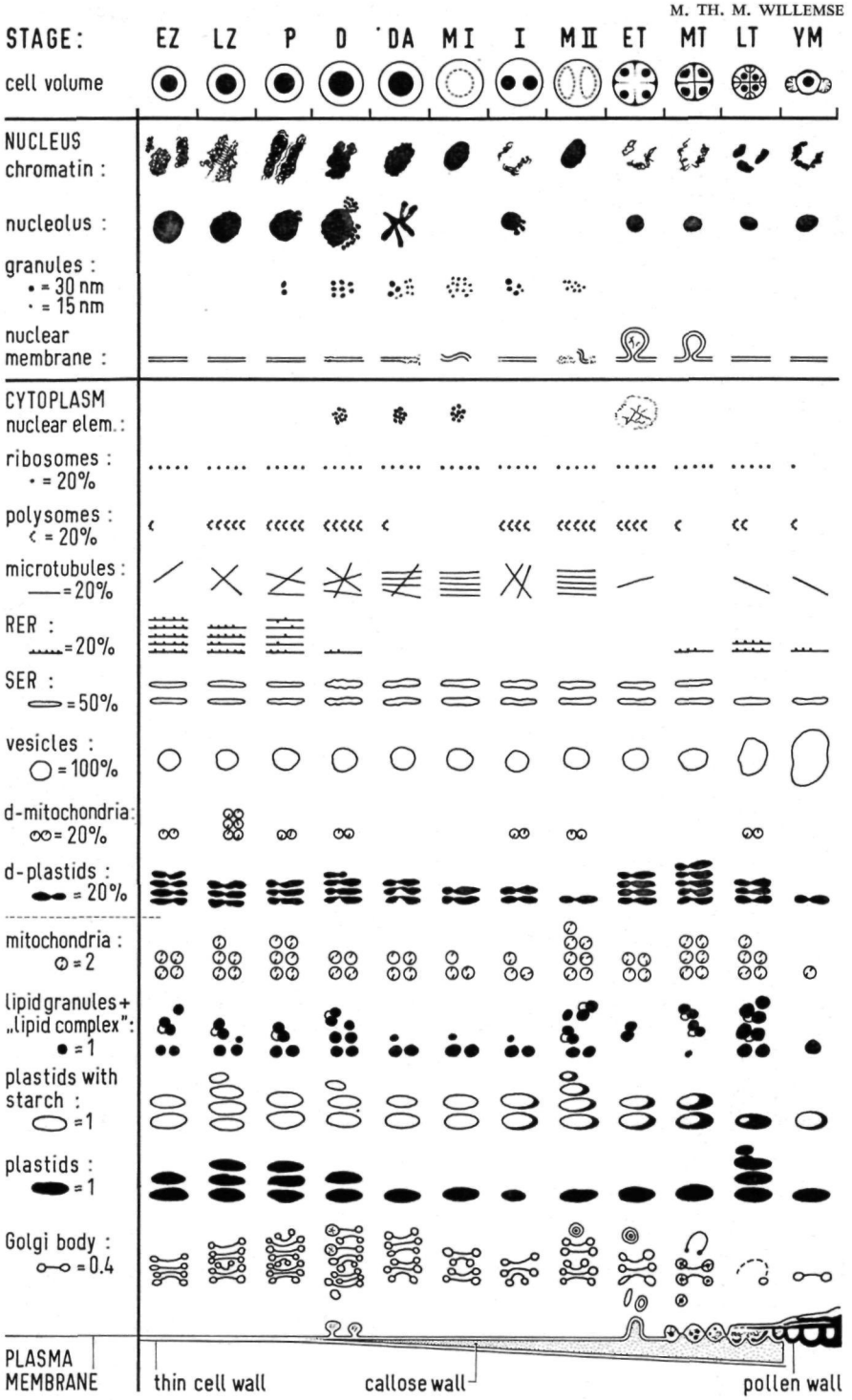
For this reason, although based on cell sections, the conclusion may suggest that starting in zygotene, the density of the cell organelle population in the cytoplasm does not change during the microsporogenesis of *Pinus sylvestris* till the young microspore stage The RER, microtubules, polysomes and the Golgi bodies are possibly exceptions, but the quantitative changes of these organelles could be related to the process of cell division or cell wall formation If a numerical stability of most cell organelles exists during microsporogenesis, it means that in the young microspore about one fourth part of the cell organelles of the diploid zygotene cell is present in each young microspore The haploid nucleus of the young microspore is situated in cytoplasm of mainly diploid origin

5 SURVEY AND SUMMARY OF THE QUANTITATIVE AND MORPHOLOGICAL CHANGES DURING MICROSPOROGENESIS OF PINUS SYLVESTRIS L

In diagram II a survey is given of the most important quantitative and morphological changes of the cell organelles and the cell nucleus

5 1 The cell size

During the meiotic divisions the cell size increases The nucleus as well as the cytoplasm enlarge Therefore, the density of cell organelles per unit of cytoplasm changes also.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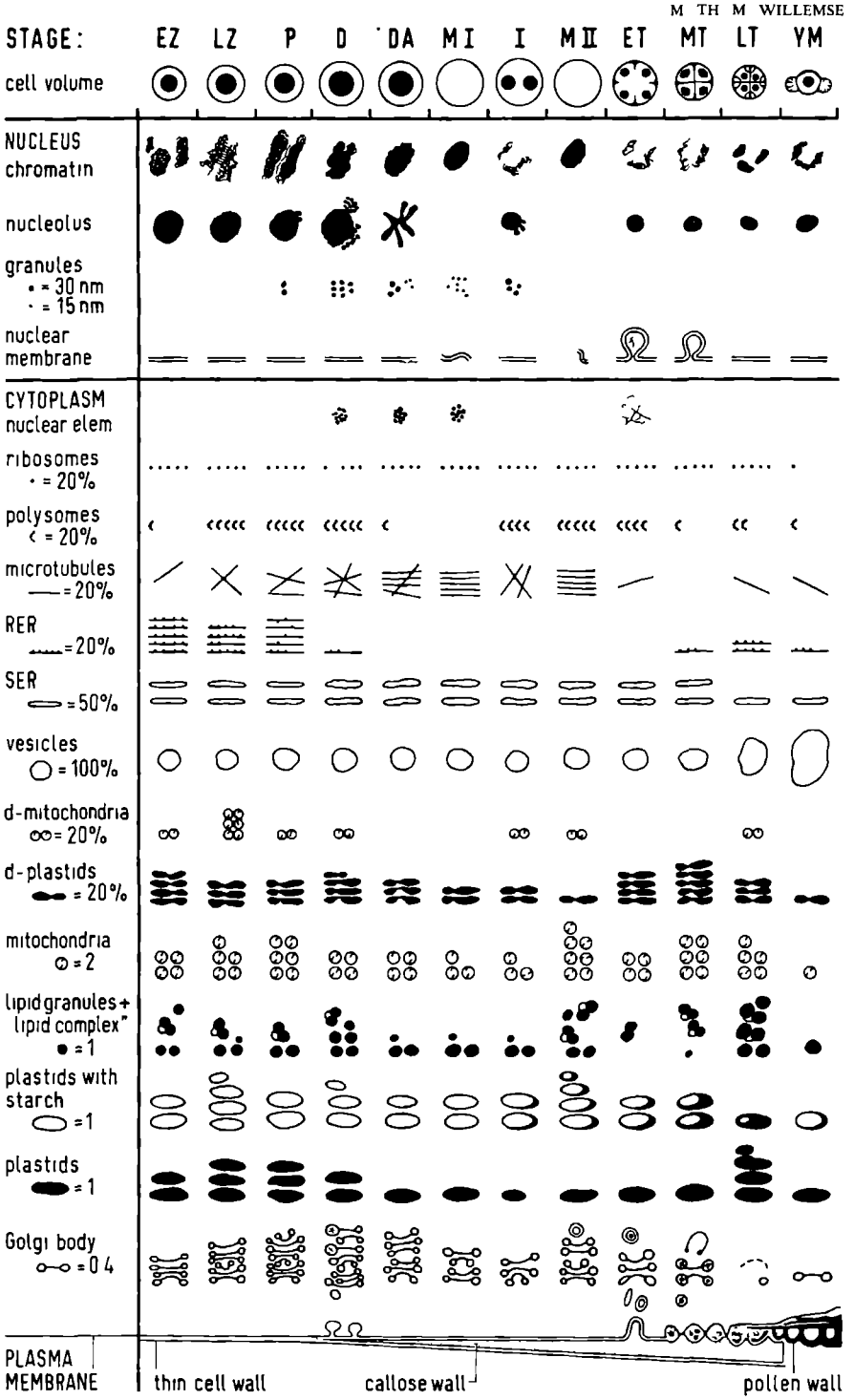
pollen wall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CYTOPLASM
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polysomes
‹ = 20%
microtubules
— = 20%
RER
= 20%
SER
= 50%
vesicles
○ = 100%
d-mitochondria
∞ = 20%
d-plastids
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lipid granules + lipid complex"
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Golgi body
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PLASMA MEMBRANE
thin cell wall
callose wall
pollen wall

Diagram 2 Survey of the morphological and quantitative changes during the microsporogenesis of *Pinus sylvestris* The results of the quantitative approach are also drawn In the margin above the dotted line the figures represent also the % of cell sections, in which the organelle is present (compare with table I) Under the dotted line the mean value of some counted organelles is approximately given per 100 point cytoplasm area (compare with diagram I) In the young microspore (YM) no quantitative result, but only the presence of the organelle is given d-Plastids, d-Mitochondria mean dumb-bell shaped plastids, mitochondria Nuclear elem means nuclear elements in the cytoplasm derived from the nucleus

5 2 The nucleus

In late zygotene and pachytene the synaptinemal complex has been observed, the karyoplasm of the late zygotene nucleus shows many "membranes" Till diplotene the nucleolus appears homogeneous, thereafter it produces granules, while dense bodies of nucleolar material are found in the cytoplasm In diakinesis the nucleolemma is the last stage before which the nucleolus disappears The granules of the nucleolus are mixed with the cytoplasm during the telophase and are supposed to be precursors for ribosomes and polysomes This phenomenon is repeated in the second meiotic division This delivery of granular material and parts of the nucleolus has also been described for mitotic cells Before the divisions the nuclear membrane is partly broken down The nuclear membrane shows invaginations during early tetrad and middle tetrad stage, which transport chromatin-like material to the cytoplasm This action of the nuclear membrane has only been reported during microsporogenesis

5 3 The cytoplasm

In the cytoplasm ribosomes are always present During diakinesis and metaphase I there may be a lower number A high number of ribosomes is observed in the centre of the cell after anaphase I and II, which are derived from the granular karyoplasm and change into polysomes Polysomes may be related to the callose wall formation and possibly the pollen wall formation

In the cytoplasm the microtubules appear in zygotene and are orientated in prometaphase I when the nuclear membrane is partly resolved In interphase II up to the early tetrad stage they are found again

RER, lying in packets, changes into SER during pachytene and appears again in the middle tetrad stage During late tetrad stage electron dense material is found along a group of membranes SER is observed in all stages, but it diminishes in the middle tetrad stage SER is difficult to distinguish from the long flat cisternae produced by the Golgi body

Vesicles with a clear membrane remain present in the cytoplasm, which may be considered as small vacuoles, since the increase in volume during the late tetrad stage takes place before vacuolisation of the cytoplasm of the young microspore

Mitochondria do not change in morphology The number of mitochondria is

fluctuating, mainly due to the changes in the cytoplasm volume

Lipid granules increase in volume during the middle tetrad stage Their number increases during diplotene and the late tetrad stage The "lipid complex" appears during zygotene and pachytene and after telophase II till the young microspore stage, when callose and pollen wall formation starts Lipid granules are possible related to the formation of the sporopollenin

The population of an types of plastids in constant in number The plastids with a starch granule start to disappear in interphase II and their number reaches the minimum during the late tetrad stage The decrease may be related to the callose wall and pollen wall formation

The number of the Golgi bodies and the morphology of their vesicles change Many vesicles of different shape are produced during diplotene, early and middle tetrad stage This production is related to the formation of the fine fibrillar material for the callose wall and the pollen wall Golgi bodies are possibly partly renewed during the second meiotic division The nuclear membrane is partly rebuilt by fusion of Golgi vesicles

The plasma membrane is involved in the formation and orientation of callose and sporopollenin

From zygotene till the young microspore stage, the Golgi bodies, microtubules, polysomes and RER show the most changes Vesicles with a clear membrane, SER and probably mitochondria show less variations These reults in *Pinus sylvestris* could not be compared with the results described in *Tradescantia* (MARUYAMA 1968), due to the great difference in appearance and morphology of these microspores

5 4 The control of the pollen wall formation

After the meiotic division every microspore contains probably one fourth part of the organelles, which are present in the zygotene cell, except the Golgi bodies, RER, polysomes and microtubules This means that a haploid nucleus functions in a mainly diploid cytoplasm Golgi bodies, plastids, lipid granules, polysomes, ER and the plasma membrane are involved in pollen wall formation Before the separation of the four nuclei in the early tetrad stage, nuclear material is transported by means of the invaginations of the nuclear membrane to the cytoplasm This process may have a function in the control of the pollen wall formation, since the invaginations persist during the middle tetrad stage This signal, or possibly another kind of signal, of the haploid nucleus on the not yet separated and mainly sporophytic cytoplasm may also explain the findings of ROGER & HARRIS (1969) They postulate a sporophytic control of pollen wall formation on base of the formation of a normal exine around a miniature pollen grain with an incomplete chromosome number

The mainly sporophytic cytoplasm executes the orders of the haploid nucleus In pollen wall formation there may be a haploid nuclear control and simultaneously a sporophytic influence via the cytoplasm and the cell organelles

In *Pinus sylvestris* the patterning of the pollen wall depends at first on callose wall formation, simultaneous with Golgi vesicle material excretion The position

of the nucleus in the cell, determined by the spindle, is also important Thereafter, the production and orientation of the sporopollenin, mainly on the plasma membrane, complete the pollen wall pattern All these elements are related to the cytoplasm, mainly of sporophytic origin, but the start of this process is initiated in early tetrad stage by the haploid nucleus, possibly by invaginations of the nuclear membrane (VASIL & ALDRICH 1970) before the separation of the four microspores (compare with FORD 1971)

These conclusions about the control of pollen wall formation are partly in agreement with the opinion recently published by HESLOP-HARRISON (1971a)

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MORPHOLOGICAL CHANGES IN THE TAPETAL CELL DURING MICRO-SPOROGENESIS OF PINUS SYLVESTRIS L.

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SUMMARY

After zygotene the tapetal cell becomes osmiophilic The electron density of the cytoplasm decreases during the early tetrad stage and increases afterwards During the young microspore stage the tapetal cell degenerates The electron density of the cytoplasm is caused mainly by the high content of ribosomes

From interphase II onwards the tapetal cell starts to produce sporopollenin In the cytoplasm pro-orbicular bodies are found The formation of orbicules seems connected with the endoplasmic reticulum and ribosomes Outside the cell the sporopollenin appears around the orbicules and as a pollen sac against the cell wall of the endothecium cells The electron dense globules on the plasma membrane may be related to the formation of sporopollenin

1. INTRODUCTION

Some morphological changes in the tapetal cell are related to the development of the microspore

During the premeiotic stage Golgi vesicles transport cell wall affecting enzymes in the tapetal cell of *Tradescantia bracteata*. During the tetrad stage the Golgi vesicles apparently contain callase for the degradation of the callose wall around the microspore (MEPHAM & LANE 1969) MIKULSKA *et al* (1969) suppose that the endoplasmic reticulum (ER) and the large quantities of enzymes in *Larix decidua* may be associated with hydrolytic processes in the tapetal cell.

In *Podocarpus macrophyllus* (VASIL & ALDRICH 1970) and in *Paeonia tenuifolia* (MARQUARDT *et al* 1968) sporopollenin containing droplets appear on the plasma membrane of the tapetal cell. In *Lilium longiflorum* (HESLOP-HARRISON & DICKINSON 1969) and in *Pinus banksiana* (DICKINSON 1971) pro-orbicular bodies are formed in the tapetal cell ECHLIN & GODWIN (1968) showed in *Helleborus foetidus* a relation of ER and ribosomes with the formation of the pro-orbicular bodies. In *Allium cepa* (RISUENO *et al.* 1969) the formation of the nucleus of the sporopollenin granules starts between the two membranes of the ER The electron dense material increases in volume to an orbicule and is carried along cytoplasmic channels to the microspore. In different species of *Oxalis* CARNILL (1967) described a deposition of sporopollenin on lipid droplets which move to the plasma membrane. In all cases the sporopollenin appears around the orbicules outside the cytoplasm of the tapetal cell. The function of the orbicules or Ubisch bodies is not clear (ECHLIN 1971)

During and after the tetrad stage lipid globuli in the tapetal cell of *Lilium*

(HESLOP-HARRISON & DICKINSON 1969) are supposed to be centres of carotenoid accumulation In the orchid *Eulopedium sandersianum* (CHARDARD 1971) the lipid granules may be associated with the degeneration of the tapetal cell In the Darwin tulip hybrid 'Apeldoorn' the production of carotenoids takes place after the tetrad stage, at the lysis of the tapetal cells the presence of anthocyanins has been demonstrated in the loculus fluid (WIERMANN & WEINERT 1969)

During the meiotic divisions of the microspore of *Paeonia* MARQUARDT *et al* (1968) have shown three subsequent mitoses of the tapetal cell without the formation of a cell wall, followed by an increase of RNA, ER and the number of mitochondria in the tapetal cell A renewal of the cytoplasm of the tapetal cell in *Pinus banksiana* has been reported by DICKINSON (1971) and in *Tradescantia bracteata* by MEPHAM & LANE (1969).

A tapetal membrane of sporopollenin surrounds the tapetum and pollen in *Ginkgo biloba* and *Taxus baccata* (PETTITT 1966), in grasses (BANERJEE 1967), and in *Pinus banksiana* (DICKINSON 1971)

The volume of the nucleus and the DNA content of the tapetal cell in *Lilium candidum* and *L henryi* increase just before leptotene The RNA content increases from leptotene up to the ripe pollen (LINSKENS & SCHRAUWEN 1968, REZNIKOVA 1971) The protein content increases gradually (REZNIKOVA 1971) The tapetal cell shows in every stage a specific protein and enzyme pattern (LINSKENS 1966) Transport of protein to the nucleus of the meiocyte during leptotene and zygotene has been demonstrated in *Rhoeo discolor* by ALBERTINI (1971). In the tapetal cell of *Lilium longiflorum* (TAYLOR 1959) the protein synthesis has been associated with the formation of the pollen wall In *Petunia hybrida* (LINSKENS 1967) and in *Lilium candidum* (REZNIKOVA 1971) the fat content of the tapetal cell increases after metaphase II

In the secretory tapetum of *Pinus sylvestris* the morphological changes in the tapetal cell show many similarities with those reported for other plants

2 MATERIAL AND METHODS

Pieces of the male cone in different stages of development of *Pinus sylvestris* were fixed for one hour in 1 % OsO_4 at 0 °C in phosphate buffer pH 7,2. After washing in water the specimens were stained for 30 minutes in 1 % aqueous uranyl acetate, followed, after washing, by a staining with 1 % aqueous $KMnO_4$ for 15 minutes After dehydration and embedding in Epon 812 sections were cut using a Porter Blumm ultramicrotome After 5 minutes staining with Reynolds lead citrate, the sections were examined with the Philips EM 300 electron microscope at 60 KV

The description of the stages of development of the tapetal cell is based on the different meiotic and postmeiotic stages of microspore development

3 RESULTS

The secretory tapetum of *Pinus sylvestris* consists of a layer of one or two cells The tapetal cells are surrounded by two layers of cells with vacuoles the fibrous layer (endothecium) and the middle layer The vacuoles of the epidermal cells contain electron dense material (*figs 1, 2, 3*)

3 1 The tapetal cell during zygotene

The nucleus of the tapetal cell has a heterogeneous nucleolus and contracted chromatine The karyoplasm contains some granules and membrane-like structures (*fig 4*)

Plastids in the cytoplasm contain a fine granular content and electron dense material between their membranes, at first as a thin line, thereafter as a globule (*fig 6*) The cristae in the mitochondria are numerous in comparison to the number in the mitochondria of the developing microspore Small lipid granules are dispersed in the cytoplasm of the tapetal cell Golgi bodies are numerous but no vesicles are secreted Some strands of rough ER (RER) are situated around the nucleus Ribosomes and polysomes are dispersed in the cytoplasm Remarkable are the very dilated membranes with an electron transparent content which surrounds a small area of cytoplasm with organelles (*fig 5*) The tapetal cell wall starts to dissolve At some places the plasmodesmata are still visible (*fig 4*)

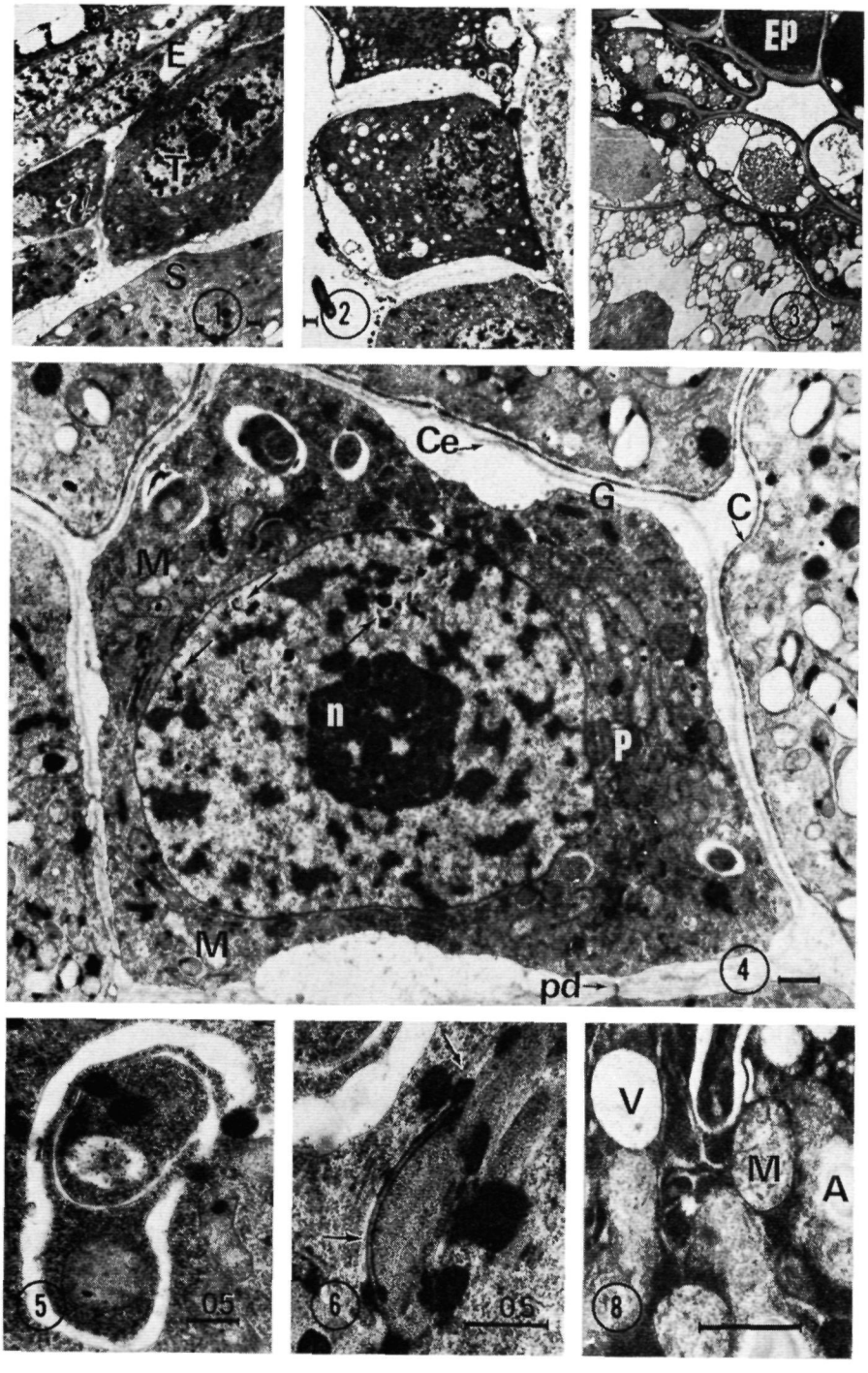
Some differences between the tapetal cell and the developing microspore could be noted A difference exists between the cell walls The nucleolus of the tapetal cell nucleus is heterogeneous, the tapetal cytoplasm is more electron dense and contains less lipid granules, less starch in the plastids and areas of cytoplasm surrounded by a dilated membrane All these features are absent in the developing microspore

3 2 The tapetal cell from pachytene up to diakinesis

The tapetal cell becomes now more voluminous and osmiophilic and may contain two or three nuclei The nucleus has a granular karyoplasm and the membrane-like structures have disappeared (*fig 7*)

The plastids have lost their electron dense globules Frequently a starch granule is observed in the plastids The mitochondria are enlarged Lipid granules are absent Large vesicles with fine granular fibrillar material become visible (*fig 8*) Golgi bodies situated in groups produce small vesicles Long strands of RER are dispersed in the cytoplasm The high number of ribosomes between the organelles makes the observation of polysomes very difficult (*figs 10, 11, 12*) The cytoplasmic areas surrounded with dilated membrane remain present

Outside the plasmamembrane large electron dense droplets are visible (*fig 7*) On the plasma membrane electron dense globular material is situated (*fig 12*) Orbicules surrounded by an irregular electron dense thin layer are sometimes found connected with the now increasingly affected tapetal cell wall (*fig 10*)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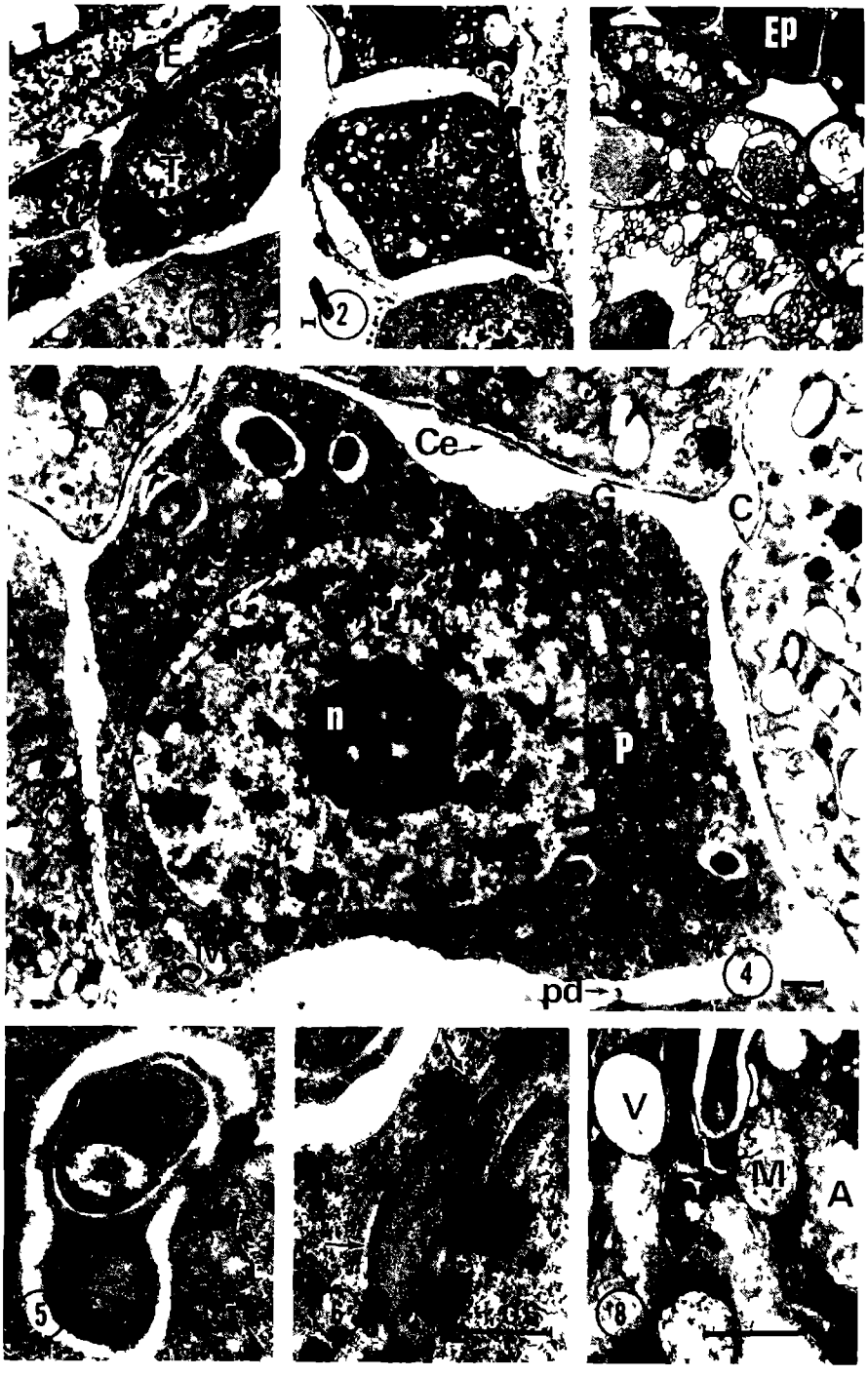
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3 3 The tapetal cell during interphase II

The Golgi bodies in the cytoplasm, still in groups, produce now more vesicles In the dilated RER fine fibrillar material is visible (*fig 11*) Excretion of electron dense globular material continues (*fig 13*) Outside the cell the orbicules increase in number (*fig 9*) The cytoplasm contains some pro-orbicular bodies with the same shape and electron density as the centre of the orbicules outside the cell (*fig 11*) The electron dense material which surrounds the orbicules appears always outside the cell

Electron dense material accumulates along the inner side of the tapetal cell wall in the direction of the endothecium cells (*fig 29*) In the locus, fragments of the tapetal cytoplasm are locally observed

3 4 The tapetal cell during the tetrad stage

During and after the second meiotic division and at the beginning of the early tetrad stage, three stages of the tapetal cell could be distinguished (*fig 2*) Firstly an osmiophilic tapetal cell, the same type as exists after zygotene, secondly a less osmiophilic cell, which corresponds to the tapetal cell as described during zygotene, thirdly an intermediate osmiophilic cell (*figs 16, 14, 15*)

In all three cell types the nucleus has a heterogeneous nucleolus The osmiophilic cell has granules in the karyoplasm (*fig 16*) The karyoplasm of the less osmiophilic cell lacks these granules, but contains a dense body (*fig 19*)

Compared with the tapetal cell during zygotene the less osmiophilic cell shows few differences Remarkable are the electron dense granules, probably pro-orbicules, sometimes associated with the RER or with ribosomes (*figs 17, 18*)

Compared with the osmiophilic cell which shows no differences with the cell after zygotene, the less osmiophilic cell has few ribosomes, fewer strands of RER, less voluminous vesicles and the Golgi bodies produce fewer vesicles (*figs 14, 16*) A transition between the less osmiophilic cell and the osmiophilic cell shows the intermediate osmiophilic cell (*fig 15*) In this last cell polysomes are present

Fig 1 Tapetal cell (T) during zygotene, around the developing microspore (S) Endothecium cells (E) and middle layer contain vacuoles, × 2,200

Fig 2 Tapetal cells during early tetrad stage, note the electron density of the cells, × 1,060

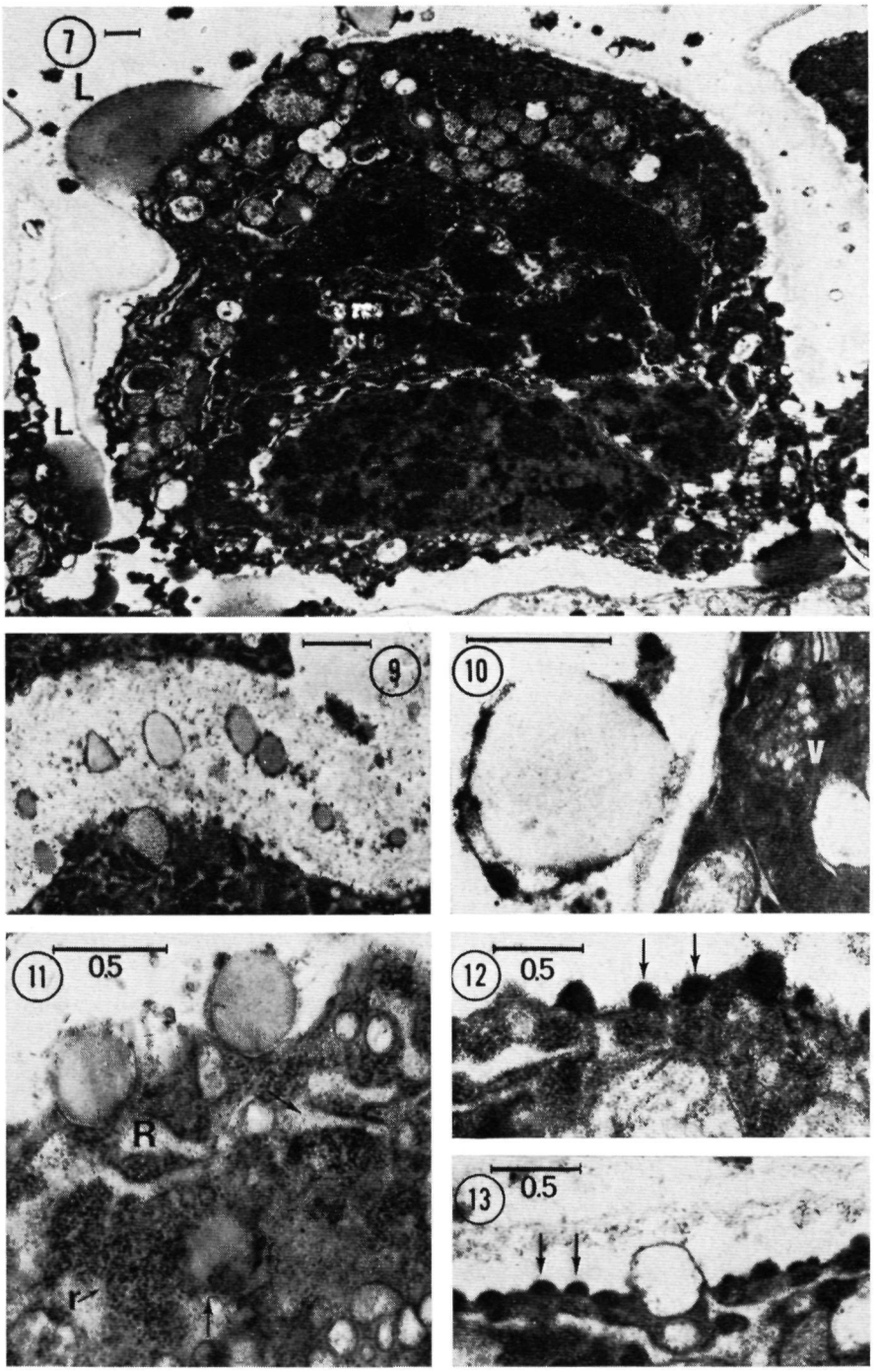
Fig 3 Degeneration of the tapetal cells Epidermal cell (Ep) with osmiophilic vacuoles, × 1,060

Fig 4 Tapetal cell during zygotene Nucleus with heterogeneous nucleolus (n) and membrane-like structures (arrows) Cytoplasm with plastids (P), mitochondria (M) and Golgi bodies (G) In the cell wall (Ce) are plasmodesmata (pd) Note the new cell wall (C) of the developing microspore, × 6,500

Fig 5 Cytoplasmic area surrounded with dilated ER, 16,500

Fig 6 Plastid with electron dense material (arrows), × 25,800

Fig 8 Tapetal cell during diplotene with dividing nucleus On the plasma membrane electron dense droplets (L), × 4,800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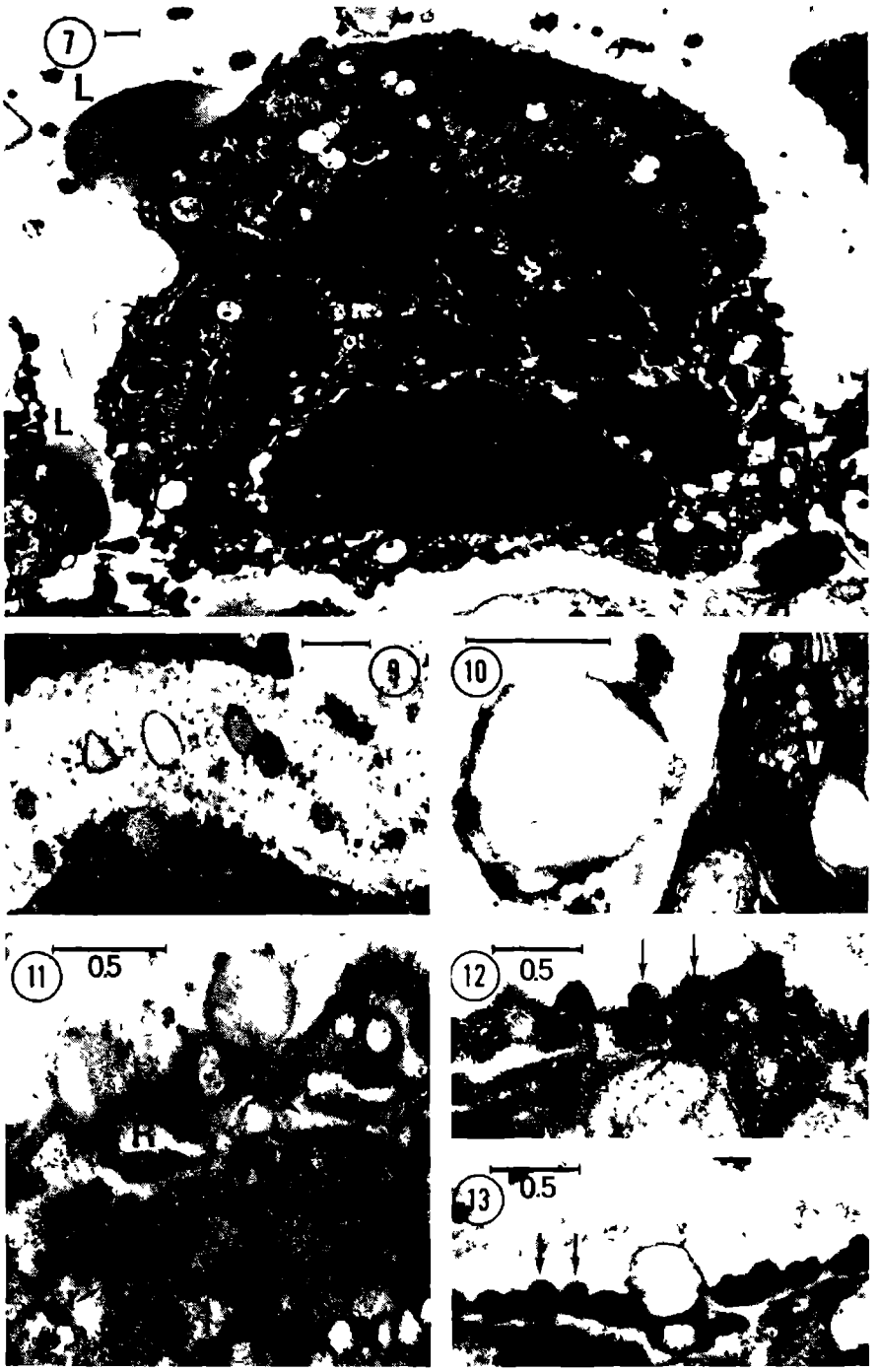
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The cell wall of the three types of tapetal cell has the same structure as the tapetal cell wall during the interphase II

When the pollen wall formation in the microspore starts, all tapetal cells become osmiophilic again and have the same appearance as during diplotene (*fig 22*) The Golgi bodies produce many vesicles A large number of accumulations of electron dense material occur on the plasma membrane (*fig 20*) The orbicules remain present, in the electron dense surrounding material lamellae of unit membrane dimension become visible (*fig 27*)

When in the late tetrad stage the pollen wall sexine and nexine I have been formed and the callose wall starts to disappear, the tapetal cell gets more vacuoles (*fig 23*) In the cytoplasm the Golgi bodies stop their vesicle production The vacuoles in the cytoplasm originate probably from the large vesicles Along the plasma membrane many orbicules are observed Electron dense material increases around the cell (*fig 21*)

No similarity shows the cytoplasm of a degenerating microspore within a tetrad with the tapetal cell The degenerating microspore is osmiophilic, although not due to the ribosomes but mainly by the electron dense material around the plastids and mitochondria (*fig 24*)

3 5 The tapetal cell during the young microspore stage

After the breakdown of the callose wall around the tetrad, the tapetal cell degenerates quickly

The nucleus remains surrounded by the nuclear membrane, the nucleolus is still recognizable (*fig 25*) All cell organelles are swollen and have an electron transparent content Some plastids contain a starch granule, the remnants of the mitochondria have an accentuated membrane (*fig 25*) Locally ribosomes remain visible (*fig 26*) The cell wall is absent, the plasmamembrane remains intact

Outside the cell membrane the orbicules are present (*fig 26*) The electron dense sporopollenin coat contains lamellae of unit membrane dimension (*fig 28*) Against the cellulose wall of the endothecium cells borders the electron dense layer of the pollen sac (*fig 30*)

After the young microspore stage the tapetal cell disappears completely Sometimes osmiophilic remnants of the tapetal cell remain visible between the microspores

Fig 7 Detail Fig 7 Cytoplasm with plastids with a starch granule (A), mitochondria (M) and vesicle (V), × 14,700

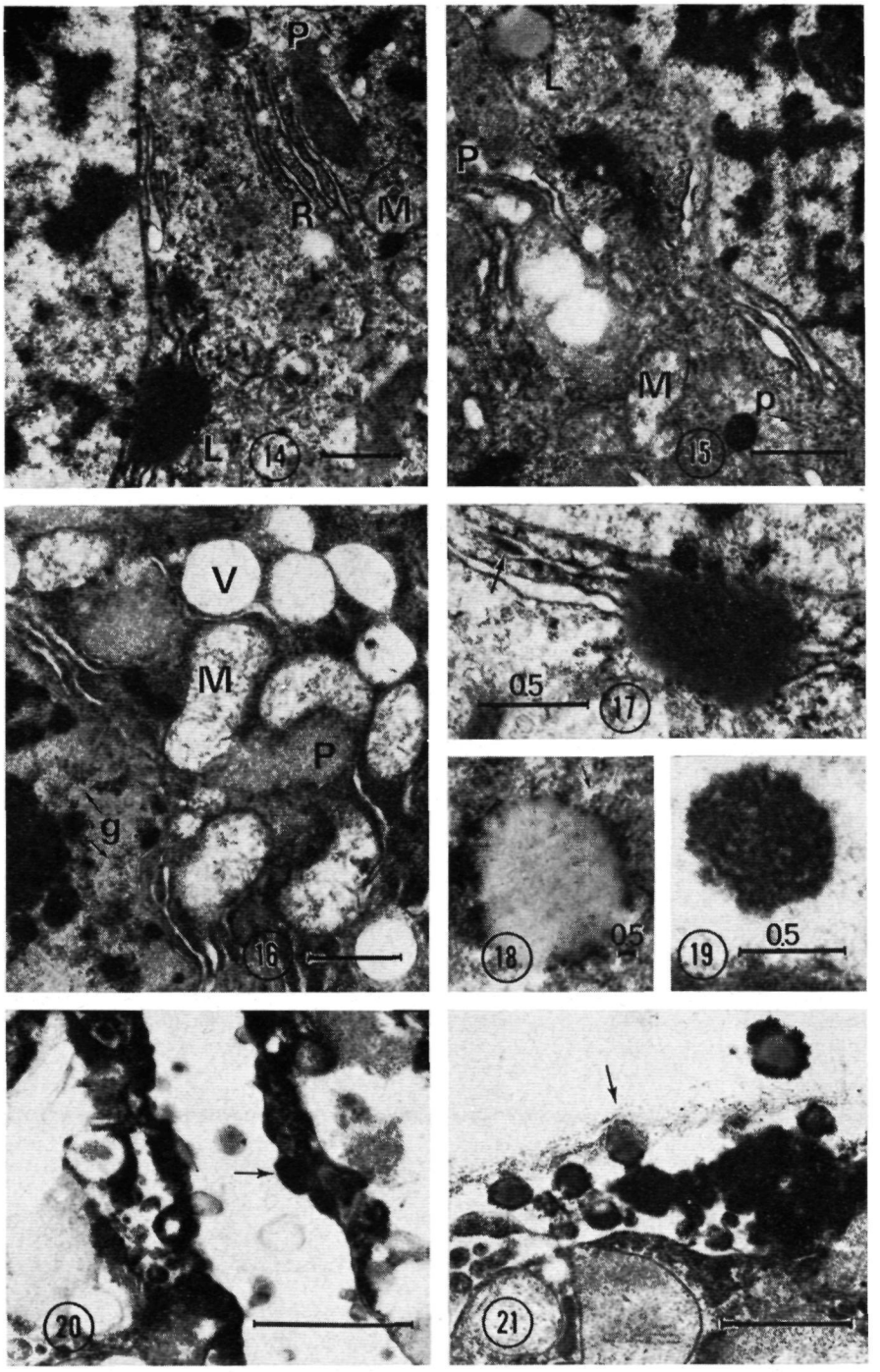
Fig 9 Orbicules between the tapetal cells, × 10,000

Fig 10 Electron dense material around the orbicule Note Golgi vesicles (v), × 20,000

Fig 11 In the cytoplasm a pro-orbicular body (arrow) Note the ribosomes (r) and the dilated RER with fine fibrillar material (arrow), × 32,000

Fig 12 Electron dense globular material on the plasma membrane during diplotene, × 25,000

Fig 13 Electron dense globular material on the plasma membrane during interphase II, × 25,000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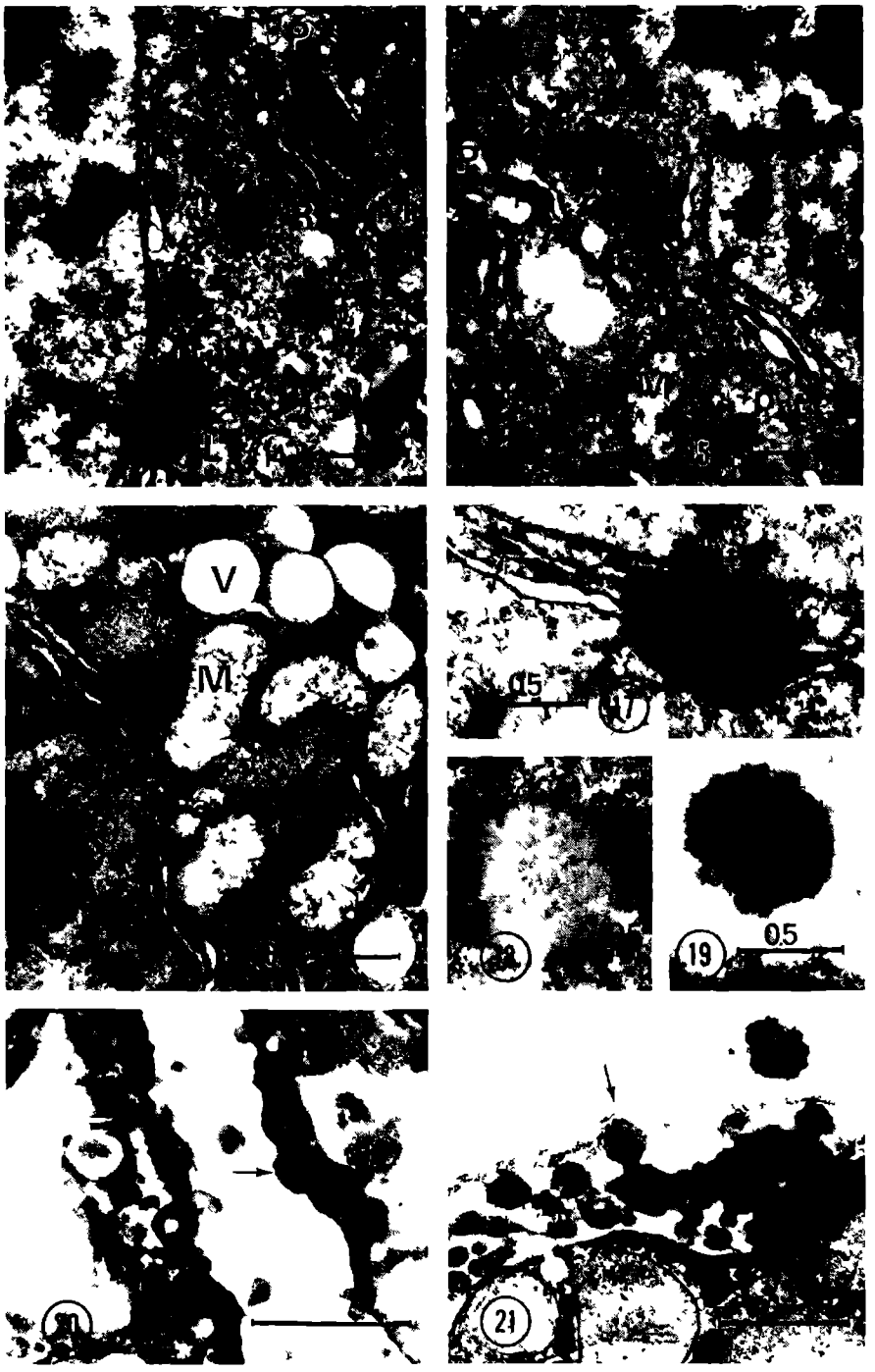
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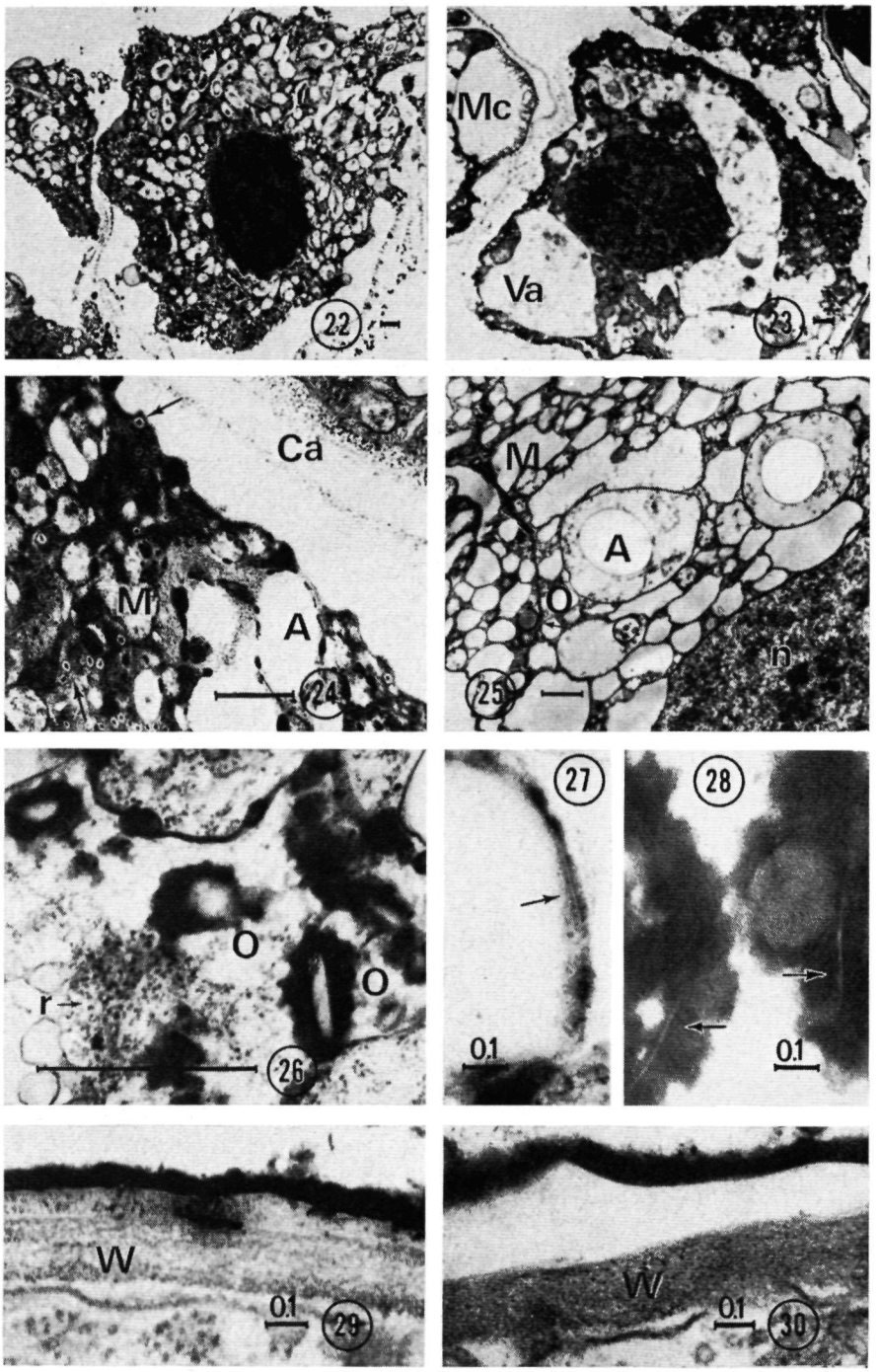
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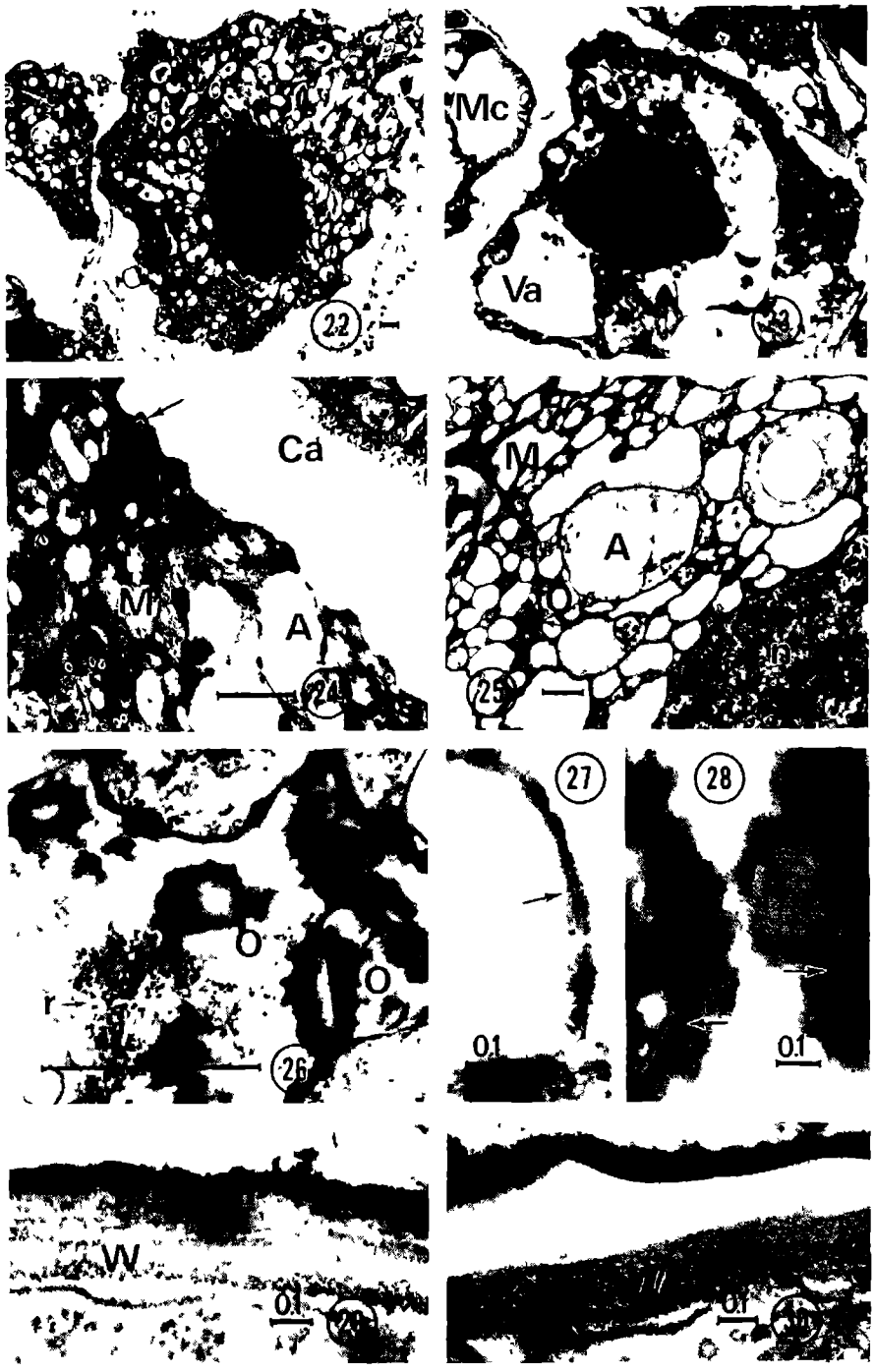
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4 DISCUSSION AND CONCLUSION

Compared with the degenerating microspore in the tetrad, the tapetal cell shows no sign of degeneration before the formation of the young microspore

After zygotene mainly the number of ribosomes increases, the strands of ER grow and the mitochondria change their number of cristae and enlarge This increase of ER, mitochondria and ribosomes has also been reported by MIKULSKA *et al* (1969), MEPHAM & LANE (1969), HOEFERT (1969), and ECHLIN (1971a) During the early tetrad stage mainly the number of ribosomes decreases and the cell becomes similar to the tapetal cell during zygotene In agreement with the report by MEPHAM & LANE (1969) and DICKINSON (1971) also a renewal of the tapetal cell of *Pinus sylvestris* takes place A division of the endothecium cell which may be the cause of a new tapetal cell, or division of tapetal cells, has not been observed Besides, the same structure of the tapetal cell wall persists For this reason, it seems that in the development of the tapetal cell two identical morphological cycles could be distinguished The first from zygotene up to early tetrad stage and the second from early tetrad stage up to the degeneration of the tapetal cell

The granular karyoplasm which appears during zygotene and after the early tetrad stage and the dense bodies in the karyoplasm during the early tetrad stage

Fig 14 Early tetrad stage less osmiophilic cell Cytoplasm with mitochondria (M), plastids (P), RER (R) and pro-orbicule (L), × 11,000
Fig 15 Intermediate osmiophilic cell The RER dilates (R), pro-orbicule (L) with ribosomes Note the polysomes (p), × 13,000
Fig 16 Osmiophilic cell Vesicles appear (V), karyoplasm with granules (g), × 13,000
Fig 17 Detail pro-orbicular body with RER Between the membranes electron dense material (arrow), × 29,000
Fig 18 Pro-orbicular body with ribosomes (arrow), × 32,000
Fig 19 Dense body in the karyoplasm of the less osmiophylic cell, × 29,000
Fig 20 Electron dense material on the plasma membrane during the tetrad stage (arrow), × 22,000
Fig 21 Electron dense material along the cell during break out of the microspores Orbicule seems to penetrate in the tapetal cell wall (arrow), × 17,000
Fig 22 Tapetal cell during the tetrad stage, × 2,200
Fig 23 Tapetal cell during the late tetrad stage, vacuoles appear (Va), Mc microspore, × 2,200
Fig 24 Degeneration of a microspore within the tetrad Plastids with a starch granule (A) and mitochondria (M) surrounded with osmiophilic material, Golgi vesicles remain visible (arrow) Ca callose wall, × 11,000
Fig 25 Degenerating tapetal cell during the young microspore stage Plastids with a starch granule (A), mitochondria (M) and the nucleolus (n) remain recognizable, × 5,600
Fig 26 Orbicules in the tapetal fluid (0), note the ribosomes (r), × 31,000
Fig 27 Lamellae of unit membrane dimension in the growing orbicule (arrow), × 57,000
Fig 28 Lamellae of unit membrane dimension remain visible in the orbicules (arrows), × 67,000
Fig 29 Interphase II pollen sac appears against the endothecium wall (W), × 56,000
Fig 30 Pollen sac during the young microspore stage W Cell wall of the endothecium cell, × 56,000
Unless mentioned otherwise, the line on the figures represents a length of 1 μm

may be related to the formation of ribosomes, as occurs during diplotene and diakinesis in the developing microspore of pine (WILLEMSE 1971) The high amount of ribosomes, probably polysomes, may be related to the protein synthesis in the tapetal cell (LINSKENS 1966, REZNIKOVA 1971) Polysomes are mainly observed in the intermediate cell

The production of Golgi vesicles takes place after zygotene and the early tetrad stage It could not be demonstrated that the production of Golgi vesicles is related to the formation of cell wall affecting enzymes, although during the production of the vesicles the tapetal cell wall and the callose wall around the microspore disappear

As in *Helleborus* (ECHLIN & GODWIN 1968) the formation of pro-orbicules in *Pinus sylvestris* may have a relation to RER and ribosomes Outside the cytoplasm the pro-orbicules are surrounded by sporopollenin in which lamellae of unit membrane dimension are present on which the sporopollenin may be formed (ROWLEY & SOUTHWORTH 1967)

The electron dense material on the plasma membrane may contain sporopollenin and/or a carotenoid (WIERMANN 1970), but in another state than that around the orbicules Around the tapetal cell the same reaction occurs on UV radiation as in the exine of the microspore The electron dense material on the plasma membrane is probably the source of the fluorescence (WILLEMSE 1971a) The large accumulations of electron dense material on the plasma membrane disappear completely Finally, it is remarkable that the sporopollenin around the orbicules, of the pollen sac and of the sexine of the pollen wall after the tetrad stage grows while the sporopollenin around the electron dense globular material does not Whether the production of electron dense globules between the membranes of the plastids is connected with the formation of the electron dense material on the plasma membrane is not clear Electron dense material on the plastids has been also reported by MARQUARDT *et al* (1968) and ECHLIN (1971a)

The quick increase of the sporopollenin on the orbicules, pollen sac and pollen wall indicates a very high content of sporopollenin precursors in the tapetal fluid This non-electron dense material may be produced by the microspore and the tapetal cell Outside the cell the sporopollenin appears as electron dense material mainly on membranes (WILLEMSE 1971b) The last steps in the formation of sporopollenin apparently occur in the tapetal fluid, as WIERMANN (1970) has shown in the synthesis of flavonol and anthocyanidin The tapetal fluid plays an important role in the relation between the developing microspore and the tapetal cell (ROWLEY 1963)

A similarity between the tapetal cell and the developing microspore of *Pinus sylvestris* based on the morphology of the cells exists These are the presence of membrane-like structures in the karyoplasm during zygotene, the heterogeneous nucleolus in relation to the renewal of the ribosome population and the formation of sporopollenin

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The author is much indebted to Prof Dr H F Linskens for his stimulating interest, to Dr. M M A Sassen for his critical reading of the manuscript, to Dr P van Gijzel and Dr G W. M Barendse for the translation and correction of the manuscript The author is grateful to Mrs E A J Derksen for her critical corrections

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CHANGES IN THE AUTOFLUORESCENCE OF THE POLLEN WALL DURING MICROSPOROGENESIS AND CHEMICAL TREATMENTS

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SUMMARY

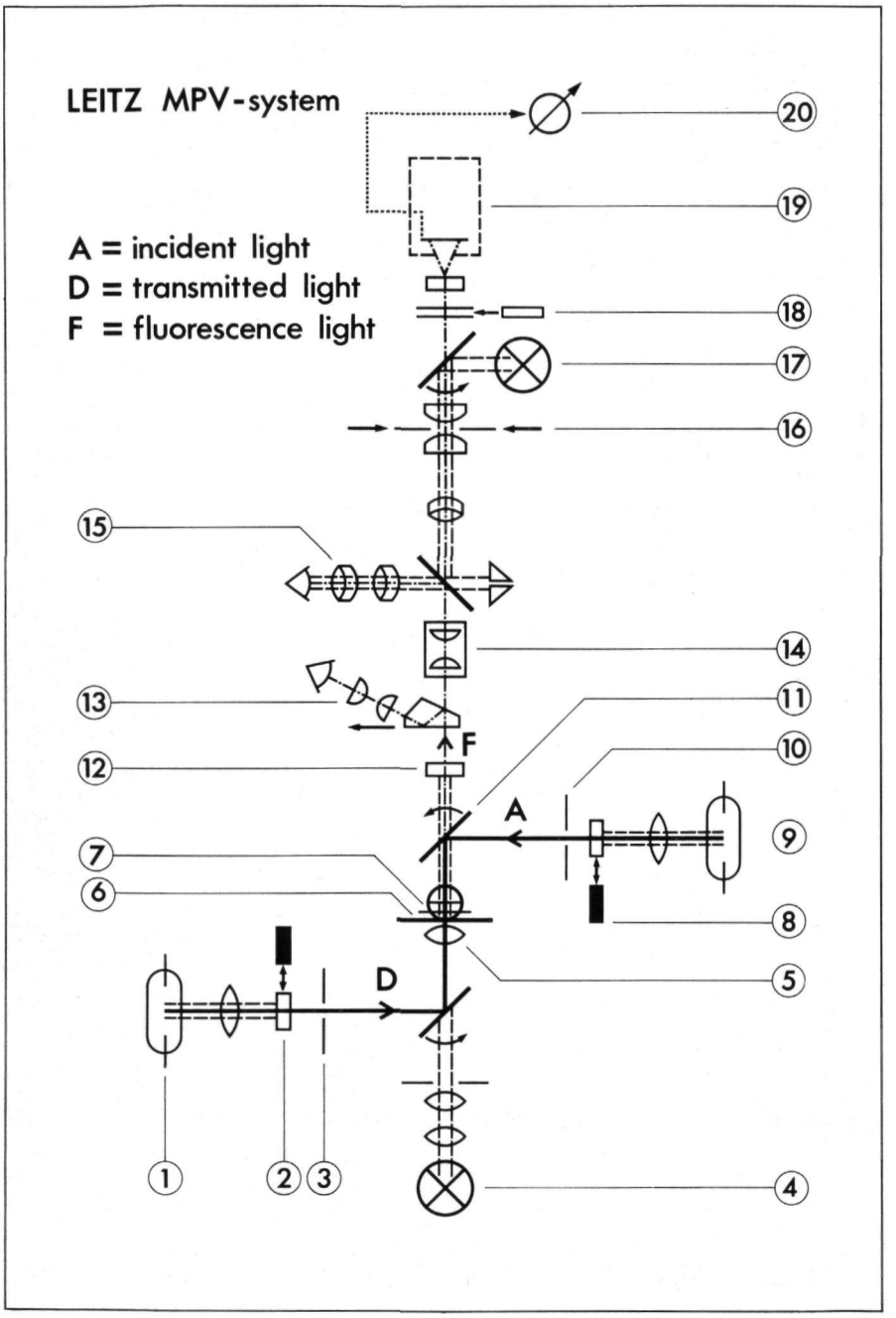
Measurements were made of the spectral maximum, the intensity, and fading of the autofluorescence spectrum during microsporogenesis and of pollen after some chemical treatments in *Gasteria*, *Pinus*, *Physostegia*, and some other plants

Mainly the sporopollenin of the sexine and the footlayer of the pollen wall showed autofluorescence which appeared to be specific for each pollen species The cytoplasm showed sometimes autofluorescence during the initiation of the pollen wall formation The spectral maximum increased in wave-length and the percentage of fading decreased before the formation of the intine, but also when the intine of the pollen wall is removed

The exine of the pollen wall of *Pinus* disappeared gradually after treatment with a mixture of acetic acid and hydrogen peroxyde The pollen wall of *Gasteria* showed a higher resistance to the chemical treatments compared with *Pinus* pollen

1 INTRODUCTION

In plants many cytoplasmic components, cell wall compounds and pollen grains show UV-fluorescence (GOODWIN 1953) BERGER (1934) and ASBECK (1955) described the colour of fluorescence of various pollen species Their fluorescence colour and spectrum appeared to depend on type and geological age, due to various substances in the pollen wall (VAN GIJZEL 1967, 1971a) Changes in fluorescence spectrum during pollen development of *Pinus sylvestris* and *Gasteria verrucosa* have been observed (WILLEMSE 1971a) In the exine of the pollen of *Ambrosia trifida* a difference could be distinguished between the endexine and extexine by measuring the UV absorption spectra (SOUTHWORTH 1969) WATERKEYN & BIENFAIT (1971) reported that the footlayer and the nexine II show a less intensive secondary fluorescence than the sexine of the developing pollen wall of *Lilium* sp and of *Ipomoea* These differences may be due to the changes in the sporopollenin content or to the appearance of other products in the exine The exine is chemically composed of a substance named sporopollenin, an oxidative polymer of carotenoids and/or carotenoid esters (BROOKS & SHAW 1968, SHAW 1971) The constitution of the microspore wall of *Lycopodium clavatum* and *Pinus sylvestris* was more extensively investigated by SHAW & YEADON (1966) In both plants the microspore walls consist of 10–15% cellulose, 10% xylan, 10–15% of a lignin-like fraction and a lipid fraction of 55–65% In the pollen wall of *Pinus sylvestris* also callose is present (MARTENS *et al* 1967)


LEITZ MPV-system
A = incident light
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F = fluorescence light
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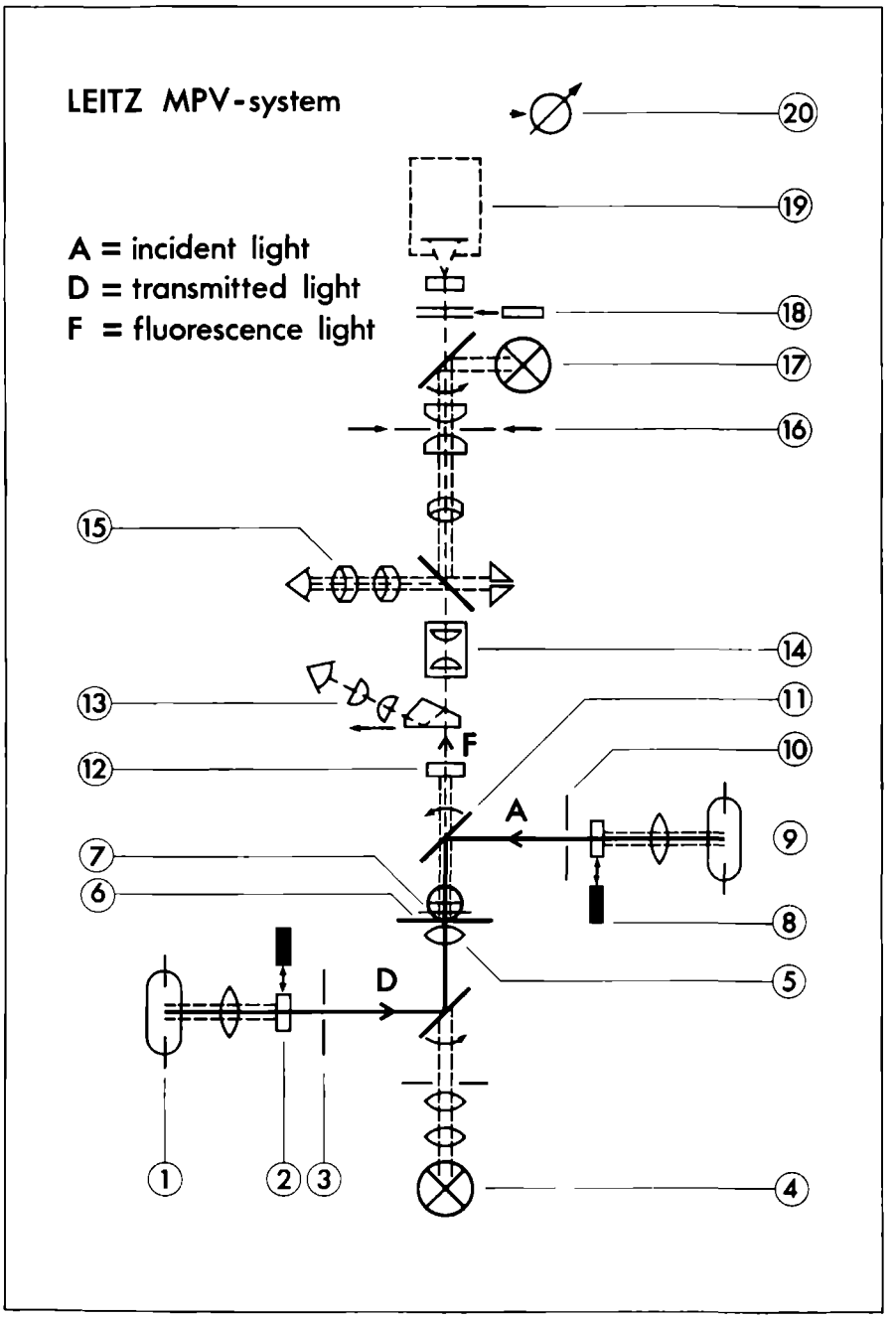
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More details on the chemical composition of the lipid components of the microspore wall of *Lycopodium clavatum* and *Pinus pinaster* are given by DUNGWORTH *et al.* (1971).

After chemical treatments of fresh pollen, the autofluorescence may change in colour, which is caused mainly by changes in the composition of the pollen wall (VAN GIJZEL 1971a; WILLEMSE 1971a). Subject of this study are the changes in fluorescence during pollen development and under influence of chemical treatments of pollen combined with the morphological changes in the pollen wall.

2. MATERIAL AND METHODS

An investigation of the changes in the autofluorescence, in the following called fluorescence, was made during the pollen development of *Gasteria verrucosa* (Mill.) Haw. (Liliaceae), *Physostegia virginiana* (L.) Benth. (Lamiaceae) and a number of other plants (see Diagram I), of which some developmental stages were measured. All plants originated from the Botanical Garden of the University of Nijmegen. For the various chemical treatments mainly pollen of *Pinus pinaster* Ait., stored at −10 °C, and fresh *Gasteria* pollen were used.

The fluorescence microphotometry was carried out with the Leitz MPV-system mounted on an Orthoplan microscope, see figure.

The main excitation wave-length is 365 nm, which was obtained by means of a stabilized high pressure HBO 100 lamp and the UG 1 (4 mm) and BG 38 excitation filters. At transmitted illumination a dark field oil condensor was used with glycerin-phosphate buffer pH 7.2, 1:1 as oil immersion. Furthermore, an objective lens Pl 40/0,65 and a barrier filter K 430 were used. The diameter of

◁

Leitz MPV-system after VAN GIJZEL (1971b)
1: high pressure Hg-lamp HBO 100
2: excitation filters UG 1 (4 mm) and BG 38 (4 mm)
3: diaphragm
4: Tungsten lamp 6V 5A for normal light
5: microscope condensor
6: object stage
7: objective
8: excitation filters UG 1 (4 mm) and BG 38 (4 mm)
9: high pressure Hg-lamp HBO 100
10: diaphragm
11: dichroitic mirror of illuminator after PLOEM
12: barrier filter (K 430)
13: binocular tube
14: ocular in monocular tube
15: observation ocular for measuring diaphragm
16: variable measuring diaphragm
17: illumination of measuring diaphragm
18: filter with monochromatic stages
19: photocell with photomultiplier
20: recorder or potentiometer

the variable measuring diaphragm was fixed for all measurements at 100 scale units, for pollen grains smaller than this diaphragm a correction has been applied All specimens were measured at about 23°C in a 0 05 ml drop of distilled water (pH 5 5) under a cover glass For a more detailed description of the methods of fluorescence microphotometry the reader may be referred to VAN GIJZEL (1967, 1971b)

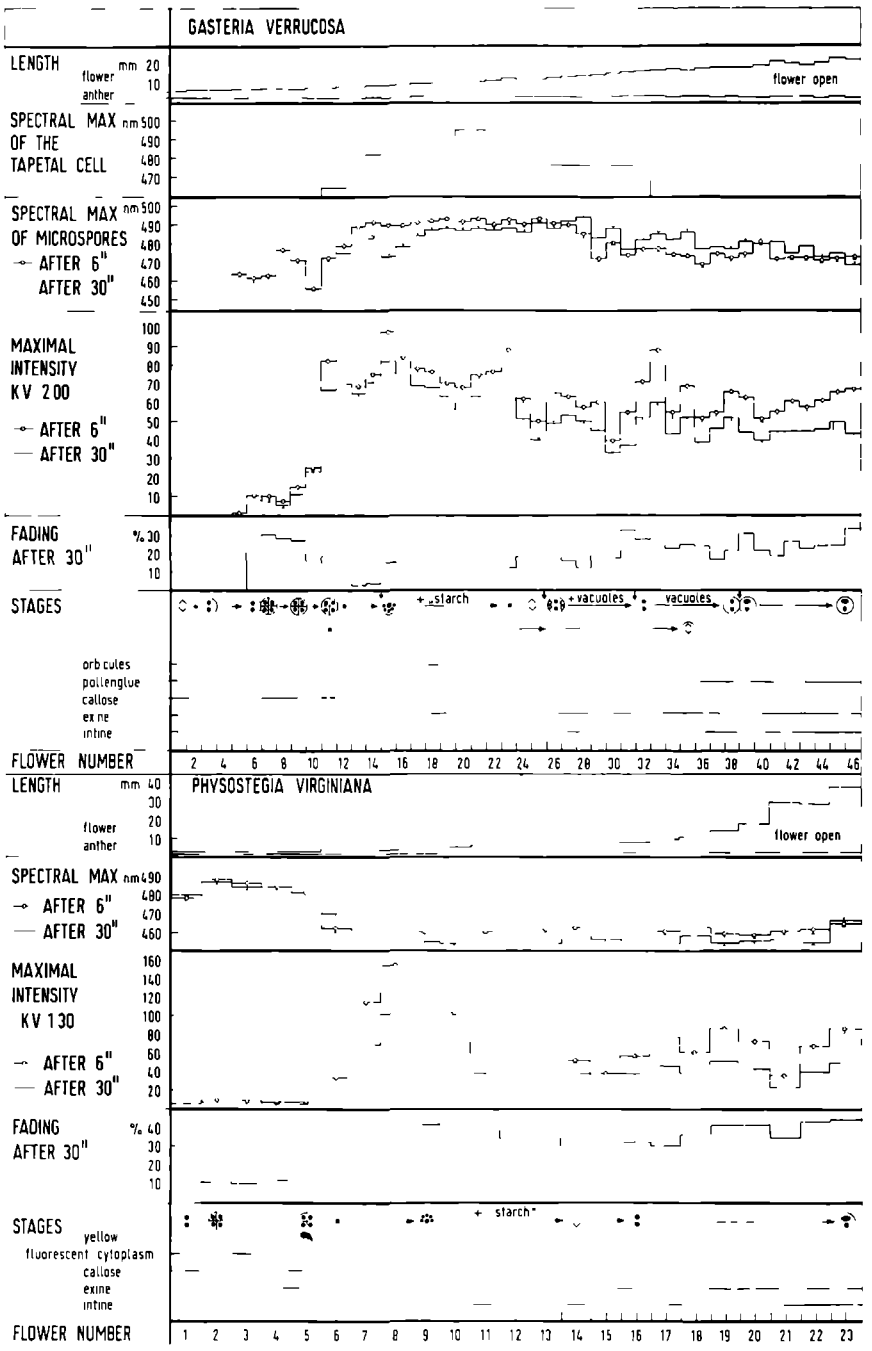
The spectral maximum and the maximal intensity in arbitrary units were recorded after 4–8 seconds (average 6″) and 27–33 seconds (average 30″) The percentage of fading or increase in intensity during 30″ was calculated, equaling the value of the intensity after 6″ = 100% In all cases the value of the intensity was calculated for 1 80 KV The mean value and standard deviation of at least three measurements were determined In all measurements the intensity of the background was less than 4% The error in the measured wave-length amounts to ± 0 5 nm

Some chemical treatments represent partly a modification of the methods used by SHAW & YEADON (1966) After homogenization all steps were boiled under reflux After cleaning in ether, ethanol 96% and water, the pollen mass was divided into several samples Each of them was treated either with 6% KOH, or with ethanolamine or with acetic anhydride and concentrated H_2SO_4 (9 1), or with acetic acid (98%) and H_2O_2 (30%) 1 1 After each treatment one or more washings with water were carried out After the treatment with KOH and the washing with water the pollen samples were cleaned with ethanol 96% and ether The medium in which the pollen was treated was also tested for fluorescence The pollen grains were measured while mounted in this medium The wings of pine pollen were measured separately

For electron microscopy the pollen grains were fixed in buffered 1% OsO_4 for 30′ at pH 7.2 After washing with water the pollen was stained with uranyl acetate in 70% ethanol for 30′ After dehydration and embedding in Epon 812 sections were cut using a Porter Blumm ultramicrotome The sections were examined by means of a Philips electron microscope EM 300 at 60 KV For light microscopy pectic substances in the pollen were stained with ruthenium red (1 5000) in water Callose was stained with a 0 005% solution of aniline blue in 50% ethanol

▷

Diagram 1. Changes in fluorescence during microsporogenesis of *Gasteria* and *Physostegia* The mean values with standard deviations are given of the spectral maximum after 6″ and 30″, of the intensity after 6 and 30 and the percentage of fading during 30″ for the different stages of microsporogenesis The standard deviation is drawn as a line only on one side of the mean value point The fluorescence of the tapetal cell of *Gasteria* has also been measured Because of the irregularity of the cell only the mean value of the spectral maximum is noted The appearance of the exine, intine, pollen glue, orbicules and also the fluorescence of the cytoplasm and the disappearance of the callose wall are shown by a line in relation to the stages of development The increase in length of the flower and the anther are also presented The length of the inflorescence of *Gasteria* is 14 9 cm, of *Physostegia* 14 5 cm, of the latter the flowers of one row only were measured


GASTERIA VERRUCOSA
LENGTH
flower
anther
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flower open
SPECTRAL MAX OF THE TAPETAL CELL
nm
SPECTRAL MAX OF MICROSPORES
AFTER 6"
AFTER 30"
MAXIMAL INTENSITY KV 200
FADING AFTER 30"
%
STAGES
+ starch
+ vacuoles
vacuoles
orbicules
pollenglue
callose
exine
intine
FLOWER NUMBER
PHYSOSTEGIA VIRGINIANA
MAXIMAL INTENSITY KV 130
yellow fluorescent cytoplasm

The infrared spectrum of the solution of acetic acid with H_2O_2, in which the pollen was treated during different periods, was measured at a frequency of 4000–800 cm^{-1} with a Perkin-Elmer 257 recording spectrophotometer for potassium bromide discs. At the start of the measurements some KBr was added to the solution.

3. RESULTS

3.1. Changes in fluorescence during microsporogenesis

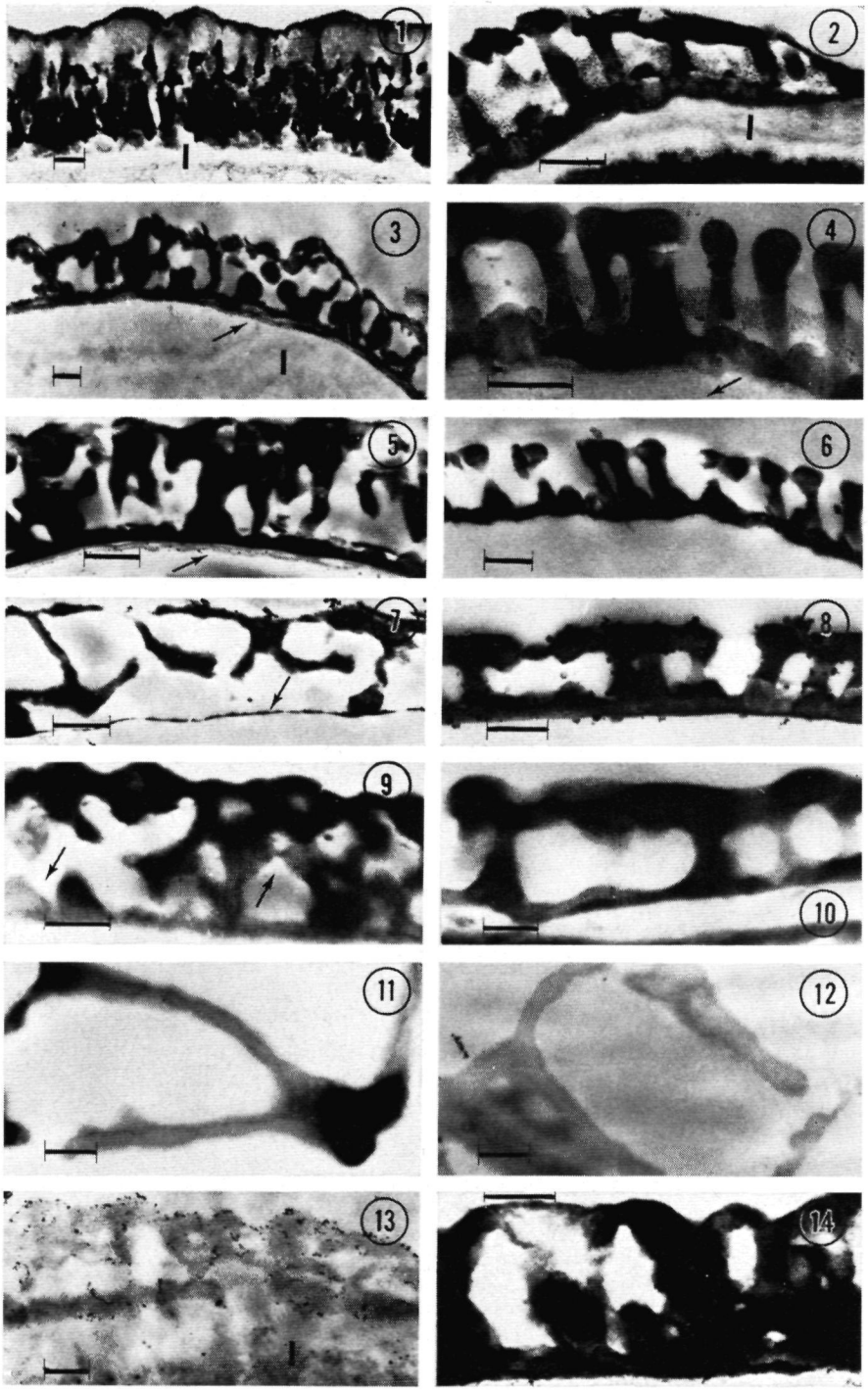
During microsporogenesis the fluorescence changes in the pollen, which has a broad fluorescence spectrum with one maximum. The racemose inflorescence of *Gasteria* and *Physostegia* permits the easy collecting of many different stages of development in a normal sequence. The results of the changes in fluorescence and in morphology are compiled in *diagram 1*.

A comparison between *Gasteria* and *Physostegia* reveals many remarkable differences in fluorescence during microsporogenesis.

The spectral maximum in *Gasteria* lies between 455–470 nm in cells in the tetrad stage, increases in wave-length after the break-out of the microspore to 480–490 nm, and decreases in wave-length around 470 nm when the intine formation starts. A shift in the spectral maximum occurs up to the beginning of the intine formation (flower 26) in the direction of 450 nm, thereafter in the

▷

Fig. 1. Pollen wall of *Physostegia*. Exine with electron dense material in less electron dense material. I = intine, × 10,000.
Fig. 2. Pollen wall of *Gasteria*. Note the material between the bacules. I = intine, × 20,000.
Fig. 3. Pollen wall of *Pinus* after treatment with ether, ethanol and water. Nexine II layered (arrow). I = intine, × 8,500.
Fig. 4. *Gasteria:* pollen wall after treatment with ether, ethanol and water. Note the affected intine (arrow), × 25,400.
Fig. 5. *Pinus:* pollen wall after treatment with KOH and washing with water. The nexine II is less electron dense (arrow), × 16,200.
Fig. 6. *Gasteria*: pollen wall after treatment with KOH and washing with water. The material between the bacula and the intine is absent, × 14,800.
Fig. 7. *Pinus*: pollen wall after treatment with ethanolamine. Note the footlayer (arrow) and disappearing bacula and tectum, × 18,500.
Fig. 8. *Gasteria*: pollen wall after treatment with KOH and washing with water, ethanol and ether, × 18,000.
Fig. 9. Pollen wall of fresh *Pinus* pollen after treatment in acetic acid – H_2O_2 during 5′. The electron dense material starts to disappear (arrow), × 19,700.
Fig. 10. *Gasteria*: pollen wall after treatment in acetic acid – H_2O_2 during 30′, × 17,000.
Fig. 11. Pollen wall of fresh *Pinus* pollen after treatment in acetic acid – H_2O_2 during 5′. Note the disappearing electron dense material in the sexine, × 14,500.
Fig. 12. Pollen wall of fresh *Pinus* pollen after treatment in acetic acid – H_2O_2 during 10′. The sexine lacks electron dense material, × 14,000.
Fig. 13. Pollen wall of *Pinus* pollen treated in acetic acid – H_2O_2. The pollen wall, without electron dense material, is setting free from the intine (I), × 13,600.
Fig. 14. Pollen wall of *Pinus* treated in acetic anhydride – H_2SO_4, × 20,500.
The line on the figure represents a length of 0.5 μm.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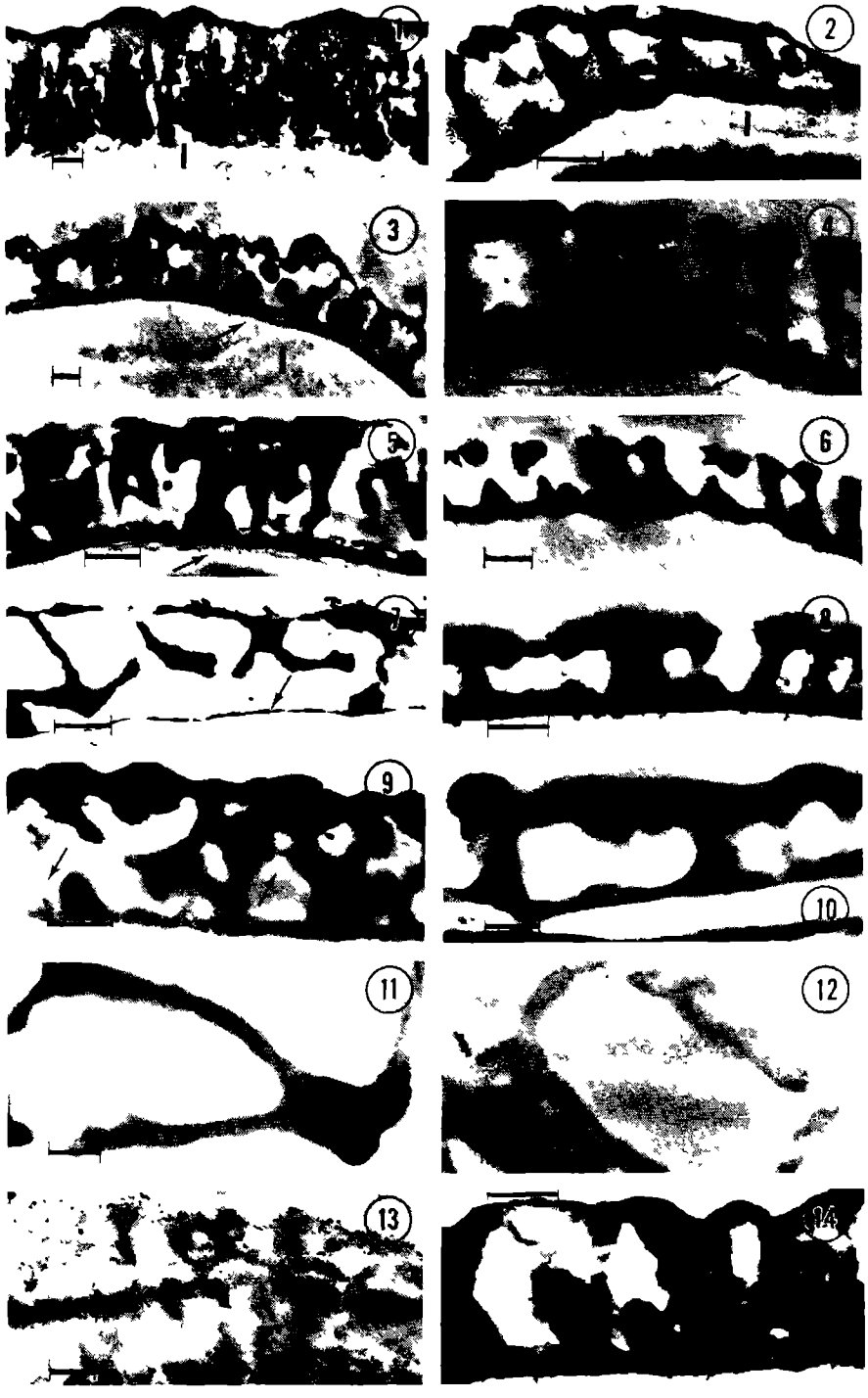
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direction of 500 nm. When the pollen is exposed for a longer time to daylight, the shift is in the direction of 450 nm (flower 46 and 23 of *Physostegia*). The mean value of the spectral maximum of the tapetal cell agrees with the mean value found in the pollen. When the intine formation starts, the spectral maximum of the tapetal cell decreases in wave-length to around 475 nm. Particularly the orbicules show fluorescence.

The intensity of fluorescence in the tetrad stage is low, but increases quickly after the break-out of the microspore. The mean value of the intensity decreases when the formation of the intine starts (flower 24), while the standard deviation shows more fluctuations. The cell stages are less synchronized. During the increase of the intensity after the break-out of the microspores, the fading percentage is low but increases slowly up to around 25%.

The intine of *Gasteria*, which lies mainly in the area of the colpus, gives a positive reaction for the presence of callose and pectine.

The cytoplasm of *Physostegia* microspore cells has a yellow fluorescent colour, which after the break-out of the microspores diminishes and finally disappears. As shown in *fig. 1*, the pollen wall contains less osmiophilic material and has an irregular shape.

During the tetrad stage the spectral maximum is about 480 nm, after break-out of the microspores it decreases in wave-length to 460 nm. The shift in the spectral maximum is mainly in the direction of 450 nm. After the tetrad stage the intensity increases. When the cytoplasm shows no more fluorescence the intensity decreases. The fading is slow during the tetrad stage. In all measurements the standard deviation is low. The intensity is much higher in *Physostegia* than in *Gasteria*.

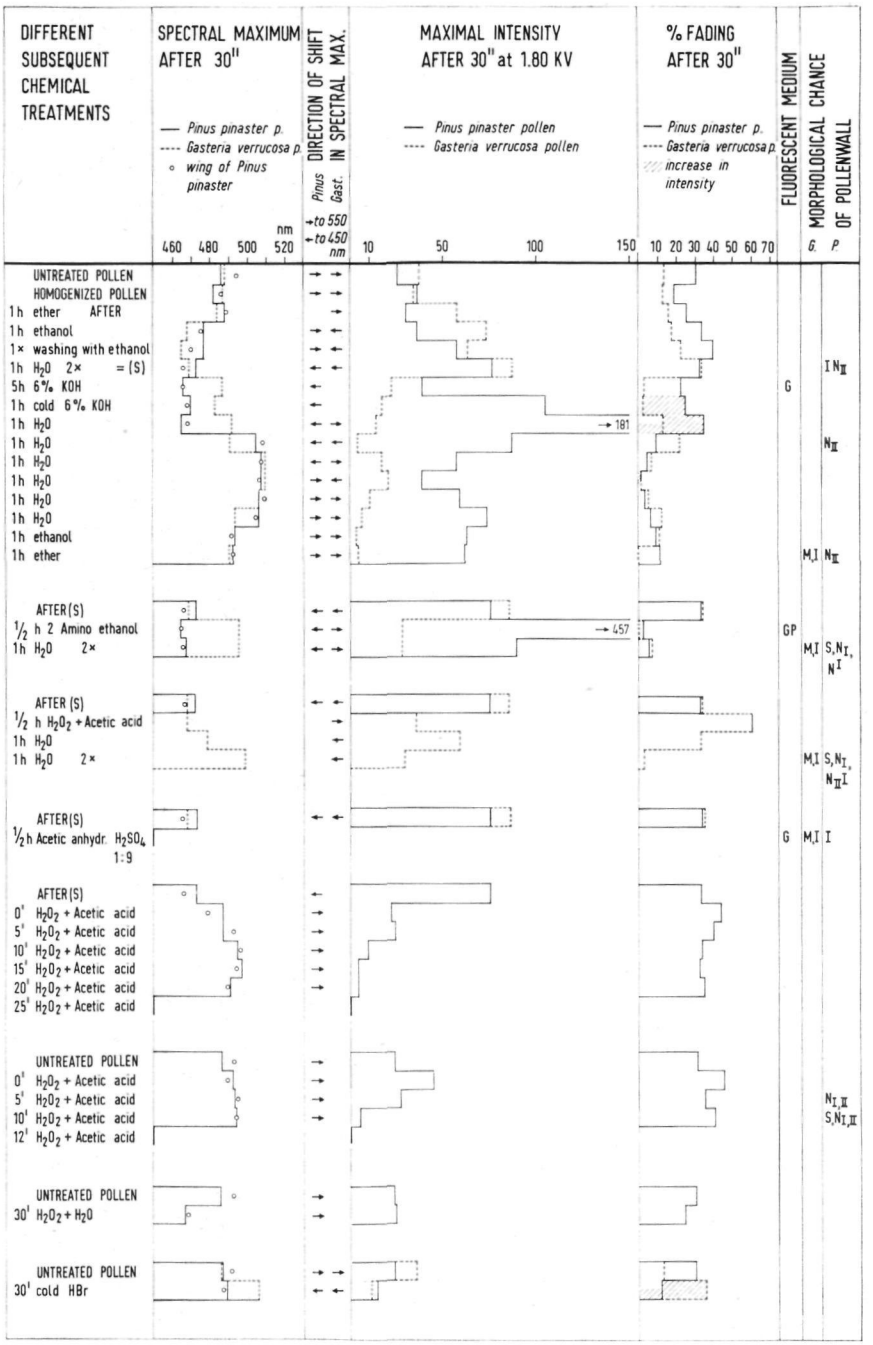
The pollen of *Physostegia* is tricolpate; the colpi areas show less fluorescence and no reaction to the aniline blue staining for callose. The areas around the colpi have an intensive fluorescence and contain callose. The whole pollen wall reacts positively to the ruthenium red stain for pectine.

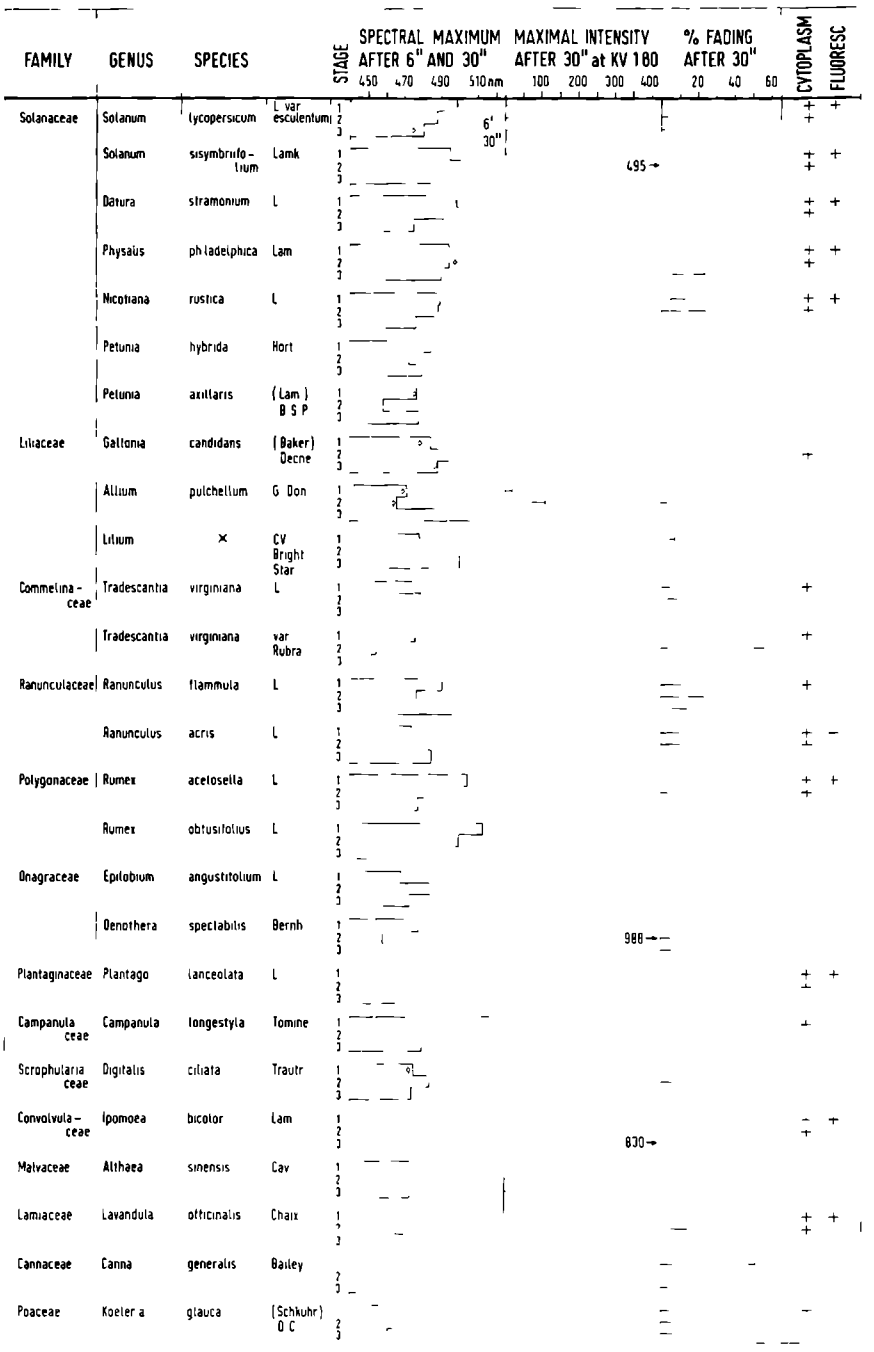
Changes in fluorescence during microsporogenesis have been investigated in some other plants as well. The whole tetrads, young microspores just delivered from the tetrads, and the ripe pollen were measured. The results are given in *diagram 2*.

Every type of pollen showed its very specific pollen wall fluorescence. During the tetrad stage the fluorescence in the pollen wall is caused by the presence of the callosic cell wall and the exine. During the young microspore stage the thickening of the exine and probably the formation of the intine give the

▷

Diagram 2. Changes in fluorescence during microsporogenesis of some plants.
The mean value is given of the spectral maximum after 6″ and 30″, of the intensity and of the percentage of fading or the percentage of increase in intensity (dotted line) after 30″ during the tetrad stage (1), during the young microspore stage (2) and of the ripe pollen (3). The standard deviations of the mean values were very low. The intensity has been corrected for 1.80 KV. The fluorescence of the cytoplasm at the different stages is also presented.


DIFFERENT SUBSEQUENT CHEMICAL TREATMENTS
SPECTRAL MAXIMUM AFTER 30"
Pinus pinaster p.
Gasteria verrucosa p.
wing of Pinus pinaster
nm
460 480 500 520
DIRECTION OF SHIFT IN SPECTRAL MAX.
Pinus
Gast.
→to 550
←to 450 nm
MAXIMAL INTENSITY AFTER 30" at 1.80 KV
Pinus pinaster pollen
Gasteria verrucosa pollen
10 50 100 150
→181
% FADING AFTER 30"
Pinus pinaster p.
Gasteria verrucosa p.
increase in intensity
10 20 30 40 50 60 70
FLUORESCENT MEDIUM
MORPHOLOGICAL CHANCE OF POLLENWALL
G P
UNTREATED POLLEN
HOMOGENIZED POLLEN
1 h ether AFTER
1 h ethanol
1 × washing with ethanol
1 h H2O 2× = (S)
5 h 6% KOH
1 h cold 6% KOH
1 h H2O
1 h H2O
1 h H2O
1 h H2O
1 h H2O
1 h H2O
1 h ethanol
1 h ether
G
I NII
NII
MI I NII
AFTER (S)
½ h 2 Amino ethanol
1 h H2O 2×
→457
GP
MI I S, NI, NI
AFTER (S)
½ h H2O2 + Acetic acid
1 h H2O
1 h H2O 2×
MI I S, NI, NII I
AFTER (S)
½ h Acetic anhydr. H2SO4 1:9
G MI I I
AFTER (S)
0' H2O2 + Acetic acid
5' H2O2 + Acetic acid
10' H2O2 + Acetic acid
15' H2O2 + Acetic acid
20' H2O2 + Acetic acid
25' H2O2 + Acetic acid
UNTREATED POLLEN
0' H2O2 + Acetic acid
5' H2O2 + Acetic acid
10' H2O2 + Acetic acid
12' H2O2 + Acetic acid
NI, II
S, NI, II
UNTREATED POLLEN
30' H2O2 + H2O
UNTREATED POLLEN
30' cold HBr


FAMILY
GENUS
SPECIES
STAGE
SPECTRAL MAXIMUM AFTER 6" AND 30"
450 470 490 510 nm
6'
30"
MAXIMAL INTENSITY AFTER 30" at KV 180
100 200 300 400
495
880
830
% FADING AFTER 30"
20 40 60
CYTOPLASM
FLUORESC
Solanaceae
Solanum lycopersicum L var esculentum
Solanum sisymbriifolium Lamk
Datura stramonium L
Physalis philadelphica Lam
Nicotiana rustica L
Petunia hybrida Hort
Petunia axillaris (Lam) B S P
Liliaceae
Galtonia candidans (Baker) Decne
Allium pulchellum G Don
Lilium × CV Bright Star
Commelinaceae
Tradescantia virginiana L
Tradescantia virginiana var Rubra
Ranunculaceae
Ranunculus flammula L
Ranunculus acris L
Polygonaceae
Rumex acetosella L
Rumex obtusifolius L
Onagraceae
Epilobium angustifolium L
Oenothera speciabilis Bernh
Plantaginaceae
Plantago lanceolata L
Campanulaceae
Campanula longestyla Tomine
Scrophulariaceae
Digitalis ciliata Trautr
Convolvulaceae
Ipomoea bicolor Lam
Malvaceae
Althaea sinensis Cav
Lamiaceae
Lavandula officinalis Chaix
Cannaceae
Canna generalis Bailey
Poaceae
Koeleria glauca (Schkuhr) D C

fluorescence In the ripe pollen the probably pigmented pollen wall with or without pollen glue causes the fluorescence

In general very few similarities are found between the investigated plants, even within the same family Two species of *Tradescantia*, *Rumex*, and *Petunia* each show some similarity in the spectral maximum, intensity and fading No relations were observed between fluorescence and the morphology or pigmentation of the pollen No distinct correlations were found between the different values of the spectral maximum, intensity and fading A distinction can be made between developing pollen with and without a fluorescent cytoplasm during the tetrad and the young microspore stage In general an increase in intensity takes place after the tetrad stage The shift in the spectral maximum is in most of the investigated pollen in the direction of 550 nm

3 2 Chemical treatments and changes in the morphology of ripe pollen

Some steps in pollen wall formation could be simulated by chemical treatment as done for ripe pollen of *Pinus pinaster* and *Gasteria verrucosa* Also the change in the pollen wall could be investigated Both fluorescence and the morphology of the pollen wall were studied during the following treatments The results of the changes in fluorescence during the different chemical treatments are given in *diagram 3*

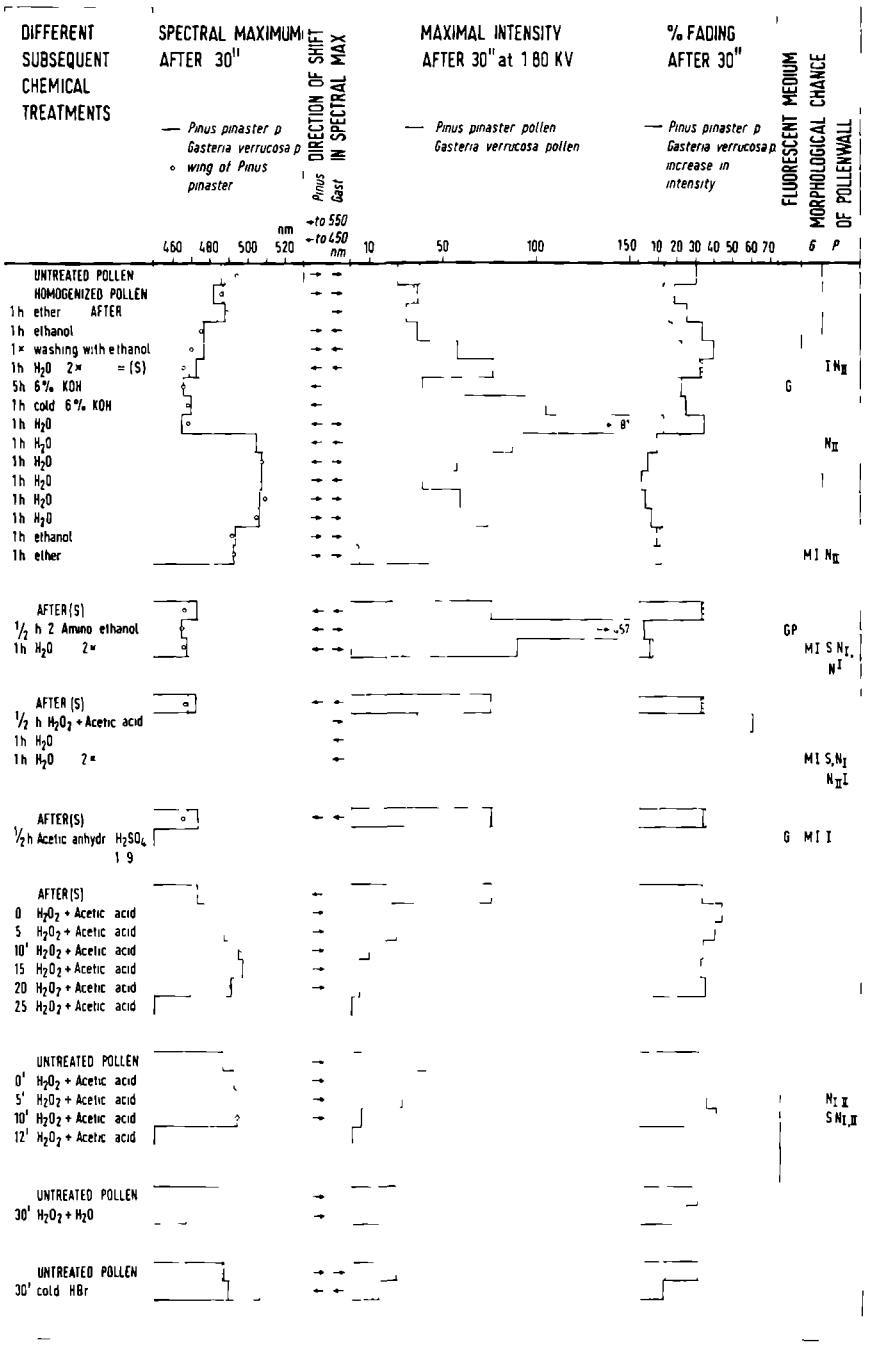
The fluorescence of the pollen wall of *Pinus* and *Gasteria* after some chemical treatments changes Between the wings, consisting of sexine only, and the pollen body of *Pinus* few differences occur It seems that the fluorescence in *Pinus* is caused mainly by the sexine Pollen of neither *Gasteria* nor *Pinus* show fluorescence of the cytoplasm

After washing with ether, ethanol and water, the spectral maximum decreases in wave-length, the intensity as well as the fading percentage increase This occurs to a greater extent in *Gasteria* than in *Pinus* The morphology of the pollen wall after these treatments shows fewer changes In *Gasteria* the intine shows a fibrillar structure compared with the normal wall (*fig 2, 4*) In the nexine II of *Pinus* some layers are clearly visible (*fig 3*) Both pollen walls react positively to the ruthenium red stain

▷

Diagram 3 Changes in fluorescence during chemical treatments of the pollen wall

Of each step of the different subsequent chemical treatments of *Gasteria* and *Pinus* pollen the value of the spectral maximum after 30 , the direction of the shift in the spectral maximum, the maximal intensity in arbitrary units after 30″ at 1 80 KV and the percentage of fading or increase in intensity (shaded columns) of the fluorescence spectrum are given The morphological changes and the fluorescence of the medium in which the pollen were treated are noted G = *Gasteria*, P = *Pinus*, S = sexine, N I and N II = nexine I and II, I = intine and M = material between the bacula of *Gasteria* Some treatments were started with untreated pollen, others with treated or cleaned pollen = (S) All treatments of the pollen took place in boiling media, except at the HBr treatment The degradation of pollen wall material of *Pinus* in acetic acid – H_2O_2 at different time intervals is given for fresh and treated pollen


DIFFERENT SUBSEQUENT CHEMICAL TREATMENTS
SPECTRAL MAXIMUM AFTER 30"
Pinus pinaster p
Gasteria verrucosa p
wing of Pinus pinaster
DIRECTION OF SHIFT IN SPECTRAL MAX
Pinus
Gast
to 550 nm
to 450 nm
MAXIMAL INTENSITY AFTER 30" at 180 KV
Pinus pinaster pollen
Gasteria verrucosa pollen
% FADING AFTER 30"
Pinus pinaster p
Gasteria verrucosa p
increase in intensity
FLUORESCENT MEDIUM
MORPHOLOGICAL CHANCE OF POLLENWALL
460 480 500 520 nm
10 50 100 150 10 20 30 40 50 60 70
G P
UNTREATED POLLEN
HOMOGENIZED POLLEN
1h ether AFTER
1h ethanol
1× washing with ethanol
1h H2O 2× = (S)
5h 6% KOH
1h cold 6% KOH
1h H2O
1h H2O
1h H2O
1h H2O
1h H2O
1h H2O
1h ethanol
1h ether
8"
I NII
G
NII
MI NII
AFTER (S)
1/2 h 2 Amino ethanol
1h H2O 2×
>57
GP
MI S NI, NI
AFTER (S)
1/2 h H2O2 + Acetic acid
1h H2O
1h H2O 2×
MI S,NI NIIL
AFTER (S)
1/2 h Acetic anhydr H2SO4 1 9
G MI I
AFTER (S)
0 H2O2 + Acetic acid
5 H2O2 + Acetic acid
10' H2O2 + Acetic acid
15 H2O2 + Acetic acid
20 H2O2 + Acetic acid
25 H2O2 + Acetic acid
UNTREATED POLLEN
0' H2O2 + Acetic acid
5' H2O2 + Acetic acid
10' H2O2 + Acetic acid
12' H2O2 + Acetic acid
NI II
S NI,II
UNTREATED POLLEN
30' H2O2 + H2O
UNTREATED POLLEN
30' cold HBr

3 2 1 The influence of pH on the fluorescence of pollen

At first the influence of pH on the fluorescence of *Pinus* and *Gasteria* pollen at 23°C was checked A gradual change of the pH from 1 2 up to pH 11 2 (obtained from acetic acid and/or KOH) had no effect on the fluorescence spectrum At pH 13 5 (= 6% KOH) the value of the intensity doubled in *Pinus*, the percentage of fading of −20% changed into an increase of intensity of +7%, the spectral maximum shifted from 490 to 480 nm At pH 13 5 *Gasteria* pollen show no increase in intensity, the percentage of fading of −20% changed in an increase in intensity of +7%, the spectral maximum changed from 480 to 530 nm Only pH 13 5 affects the fluorescence of both pollen species

3 2 2 Treatment with KOH and with ethanolamine

During treatment with 6% KOH, water, ethanol and ether, in both kinds of pollen the value of the spectral maximum shifted to 505 nm, whereas the percentage of fading decreases In *Pinus* the intensity increases for a short while and there is no fading In *Gasteria* a strong decrease in intensity occurs The treatments with ethanol and ether result in few changes in the pollen wall of both species The morphology of the pollen wall changes in both In *Gasteria* the intine is no more observed while the exine remains unaltered after the complete treatment The material between the bacula has disappeared (*fig 6, 8*) After washing with water in *Pinus* the nexine II is affected and the intine has partly disappeared (*fig. 5*) The sexine and nexine I show the normal morphology after the treatment with ether, the nexine II has disappeared

After the treatments the pollen wall of both species shows no reaction to the staining on pectine and callose The probably total disappearance of the intine in both species may cause the increase in wave-length of the spectral maximum and the decrease of the percentage of fading

The effect of the treatment with ethanolamine on the fluorescence of the pollen wall is comparable with that of KOH The spectral maximum and intensity increase, whereas the fading decreases Morphological observations show that the intine resolves for a large part In *Gasteria* the exine does not change In *Pinus* the nexine II has disappeared and the nexine I and sexine are strongly affected (*fig 7*)

3 2 3 Treatment with H_2O_2-acetic acid

In *Gasteria* the effect of H_2O_2-acetic acid during 30″ results in an increase of the fading percentage After washing with water the spectral maximum changes in the direction of 500 nm, the fading percentage decreases The intine disappears, however, the exine has locally a low contrast (*fig 10*) It appears that the microspores of *Gasteria* and *Physostegia* during the tetrad stage totally resolve after 30″ Ripe pollen of *Physostegia* has disappeared within 30″

In *Pinus* the acetic acid-H_2O_2 mixture dissolves the pollen wall exine, only the cellulose of the intine remains intact Fresh pollen loses the pollen wall and the fluorescence after 10″, the treated pollen (= S) after 20″ In both cases the

spectral maximum increases in wave-length, whereas the intensity decreases immediately. The percentage of fading remains constant.

After 5′ the changes in the morphology of the fresh pollen become visible. The contrast containing material of the pollen wall exine starts to disappear and converts into electron transparent material This takes place first in the nexine, thereafter in the sexine (*fig. 9, 11*). After 10′ in the electron microscope the pollen wall exine lacks all contrast, but tectum, bacula, and footlayer remain recognizable (*fig 12*). This pollen wall shows no fluorescence. After 15 minutes the footlayer loses the contact with the cellulose of the intine and the exine dissolves slowly The exine is electron transparent and has a fine fibrillar structure (*fig. 13*) *Pinus* pollen treated with 15% H_2O_2 only show a decrease in the wave-length of the spectral maximum

During the treatment with acetic acid-H_2O_2 the infrared spectrum of the solution with *Gasteria* and *Pinus* pollen shows only one absorption band of 1640 cm^{-1} (6100 nm), which changes in intensity when the medium is measured after 0′, 10′, 15′ and 30′. The solution in which *Gasteria* pollen were treated shows the band of 1640 cm^{-1}, which increases in intensity up to 15 minutes and which thereafter decreases. In the solution in which *Pinus* pollen was treated, the same absorption band increases continuously in intensity. This band may represent the aromatic C = C or C = N bonds.

3 2 4. Treatment with acetic anhydride – H_2SO_4 and with HBr

The fluorescence of the pollen wall in both species disappears completely after treatment with acetic anhydride – H_2SO_4 The intine disappears, but the exine remains morphologically intact (*fig. 14*).

With HBr *Gasteria* pollen reacts with an increase in wave-length of the spectral maximum and intensity. The reaction of *Pinus* is less intensive

During all treatments the cytoplasm of the pollen gradually disappears. The standard deviation of the mean values was very low during all measurements

3 3. Spectral maxima and fading percentage of some other plant cell walls or cell wall substances

The fluorescence spectrum of the measured plant cell walls or cell wall substances has only one maximum The spectral maximum and percentage of fading after 30″ were measured in other plant cell walls and substances which may have a relation to the pollen wall. The values are given in *table 1.*

The suggestion is that the spectral maxima below the value of 480 nm are the result of polysaccharide components in the cell wall.

In preliminary experiments the pollen of *Gasteria* and *Pinus* were put in a number of solutions each of which contained one enzyme with a concentration of 1 mg/ml The fluorescence of the pollen does not change after 30′ and after 20 h, when the pollen is placed in a solution with lipase (pH 8 0, 23 C), papaine (pH 6 6, 23 °C), trypsine (pH 7 0, 23 C), pronase (pH 7.0 and 9.3, 23 °C), pepsine (pH 6.0, 30 °C), α amylase (pH 6 0, 23 °C), chitinase (pH 5 0, 30 °C), pectin esterase (pH 7 0, 23 C), pectinase (pH 4 0, 23 °C + 0 25% NaEDTA), cellulase (pH 6 6, 23 °C) With the method used no effect could be registered

Table 1. Spectral maximum and fading percentage of some plant cell walls. Callose and pectin show fluorescence, but with a very low intensity.

Material	Spectral maximum after 30″	Fading percentage after 30″
Cellulose (cell wall of *Gasteria*)	476 nm	−18
Lignine (cell wall of *Gasteria*)	477 nm	−8
Cutin (cell wall of *Gasteria*)	481 nm	−1
Callose (cell wall of *Gasteria*)	not measurable (± 475)	
Pectin (extracted from apples)	not measurable (± 475)	
β Carotene $C_{40}H_{56}$ (synthetic)	no fluorescence	
Oxidative polymer of β Carotene (data obtained from Dr. P. VAN GIJZEL)	510 nm	+57 (after 30′)

4. DISCUSSION AND CONCLUSION

The fluorescence of the pollen wall seems quite characteristic for the individual pollen species. Only within a plant family similarities may occur. The result of the treatment with HBr compared with the result in *Lycopodium* (VAN GIJZEL 1971a) shows also the specificity of microspore wall composition. Only developing pollen can be distinguished through the absence or presence of fluorescent cytoplasm. This cytoplasmic fluorescence may start during the interphase II; the relation between the formation of the pollen wall and the fluorescent cytoplasm is not yet clear.

In general, polysaccharides in the cell wall have a spectral maximum around 475 nm and a low fading percentage. The carotenoid esters may have a higher value of the spectral maximum, probably around 510 nm. During the development of the pollen the production of sporopollenin is connected with a spectral maximum of about 490 nm and a low fading. When the intine appears the spectral maximum changes to about 475 nm and the fading increases. The opposite occurs when the intine is removed by the KOH or ethanolamine treatment; then the spectral maximum increases in wave-length and the fading decreases. The orbicules of the tapetal cell show the same phenomenon. The influence of the strong alkalinity during the treatment with KOH is also repressed, probably by the resolving of intine substances. The spectral maximum does not change much compared with pollen put in a solution at pH 13.5. Elements of the intine strongly influence the fluorescence. The intensity is high after the removal or before the arrival of the intine. There may be a relation between the intensity and the content of sporopollenin, but this relation is influenced by the presence of the intine.

In ethanolamine the whole pollen wall starts to dissolve (ROWLEY & FLYNN 1966). In acetic acid-H_2O_2 first the electron density disappears and the structure remains, but shows no autofluorescence. Either the electron dense material dissolves from the nexine and sexine or the electron dense material changes into electron transparent material. The latter explanation is preferred because of the fact that the structure of the nexine I and sexine in *Pinus* does not change immediately. When the pollen wall exine starts splitting from the intine, the

material is altered and shows more fine fibrils. The change in the infrared absorption band suggests a gradual process of a loss of C = C of an aromatic ring, probably of a β-carotene (BROOKS & SHAW 1968). This means that the pollen wall sexine and nexine I may contain polymer carotenoids, which give the electron density and cause the fluorescence. The precursor of these polymer carotenoids can be electron transparent. During pollen wall formation in *Pinus sylvestris* (WILLEMSE 1971b) electron dense material, probably sporopollenin, impregnates the fibrillar material derived from the content of Golgi vesicles. This fibrillar material precipitates against the callosic wall and probably consists of polysaccharides. During the ontogeny of the pollen wall two components could be distinguished in the sexine and nexine I of *Pinus*. By the treatment with the H_2O_2-acetic acid mixture these two components are also demonstrated, probably consisting, respectively, of carotenoids and a polysaccharide which is less resistant than cellulose. The nexine II consists of a different material, because it dissolves quickly in KOH and ethanolamine. The difference between nexine II and sexine with nexine I is in agreement with the results of SOUTHWORTH (1969) and WATERKEYN & BIENFAIT (1971).

Treated pollen seems to be more resistant against the acetic acid-H_2O_2 treatment than fresh pollen. The presence of acetic acid is thereby necessary to affect the pollen wall within 30'. The pollen wall of *Gasteria* is more resistant than the *Pinus* pollen wall. The mainly callosic-pectin-like intine of *Gasteria* disappears quickly, including the material between the bacula, which may indicate that less cellulose is present. The exine of *Gasteria* is more resistant than the exine of *Pinus*. It should be noted that the fluorescence may be altered without morphological changes in the pollen wall. This can be seen from the acetic anhydride-H_2SO_4 treated pollen walls, which show an intact structure but no fluorescence, due to molecular changes only. Therefore, during preparation of pollen walls, either for electron microscopy or palynology, it can not be excluded that changes in the pollen wall occur. Even ethanol seems to have an effect on the fluorescence of the pollen wall.

Because of the complexity of the chemical composition of the pollen wall, which among others may contain sporopollenin, polysaccharides, pigments and many other substances, no explanation can be given of the causes of fluorescence on the molecular level. The unanswered questions on the change of the shift in the spectral maximum illustrate this problem. Only some relations could be indicated between the developing or affected pollen wall and the fluorescence.

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MORPHOLOGICAL AND QUANTITATIVE CHANGES IN THE POPULATION OF CELL ORGANELLES DURING MICROSPOROGENESIS OF GASTERIA VERRUCOSA

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SUMMARY

The morphological changes in the nucleus and cell organelles of *Gasteria* during the development of the pollen mother cell into the ripe pollen are described and discussed From the tetrad stage till after the first mitotic division the plastids contain electron transparent granules The number of cristae in the mitochondria increases after the first mitotic division

When the pollen wall is formed the excretion of the Golgi vesicles' content and the contact of the plasma membrane with the callose wall determine the template of the pollen wall pattern The formation of the material for the bacula, followed by that of the tectum, and finally by that for the footlayer, takes place on the plasma membrane The excretion of the fine fibrillar content of the Golgi vesicles proceeds till after the formation of the footlayer The excreted material remains between the bacula

The quantitative approach revealed that during meiosis the number of cell organelles remains constant However, an increase in number takes place first after the pollen mother cell stage and before leptotene, secondly after the first mitotic division

The data are compared with the previous results obtained with *Pinus sylvestris* (WILLEMSE 1971a, b, c)

1 INTRODUCTION

In previous studies on *Pinus sylvestris* different events during the microsporogenesis were described (WILLEMSE 1971a, 1971b, 1971c, 1971d) This study deals with the morphological and quantitative changes of cell organelles during the microsporogenesis of *Gasteria verrucosa* The different developmental stages of this plant are easy to collect (STRAUB 1937) The results will be compared with the more extensive data obtained with *Pinus sylvestris*

2 MATERIAL AND METHODS

2 1 Morphological investigations

At different stages of development of *Gasteria verrucosa* (Mill) Haw pieces of anthers were put in a buffered solution of 5% glutaraldehyde pH 7 2 at 0 °C for 22 hours After washing in the same buffer solution, the specimens were put either in 1% buffered OsO_4 pH 7 2 at 0 °C for 45′ and subsequently stained in 1% uranyl acetate for 30′, or in a buffered solution of 1% $KMnO_4$ pH 7 2 for 45′ After dehydration in alcohol the specimens were embedded in Epon 812 and sectioned with a Porter-Blumm ultramicrotome The sections prepared with

OsO_4 were poststained with lead citrate (REYNOLDS 1963). The material was examined in a Philips EM 300 electron microscope at 60 KV.

2 2. Quantitative approach

The following stages of microsporogenesis were selected: 1. pollen mother cell (PMC), 2 zygotene (Z), 3. prophase II or prometaphase II (P II), 4 early tetrad stage (ET), 5 young microspore (YM), 6. microspore in which the generative cell lies free before it starts to increase in length (M).

Countings were carried out on electron microscopical (EM) photographs of the sections of complete cells. Of one anther 20 different cells were counted The cells were fixed in $KMnO_4$ and clear photographs of cell sections in the different stages of development were selected, while in every cell section a large part of the nucleus or two nuclei had to be present.

The area of the cytoplasm and the total cell area without the cell wall were determined in the same manner as described previously (WILLEMSE 1971c) The mean values and standard deviations of the number of plastids with granules, plastids, mitochondria, lipid granules, Golgi bodies and vesicles or small vacuoles per unit of cytoplasm ($5\,9 \times 5\,9\ \mu m^2 = 100$ points) were calculated from cell sections of EM photographs at a magnification of 7,500 $\times$.

2 3. Statistical analysis

The one way analysis of variance was applied to the mean value and standard deviation of the number of counted cell organelles per unit of area of cytoplasm. If the result was significant at the 5% level, it was investigated with SCHEFFÉ's (1959) or TUKEY's test for multiple comparison for each pair of mean values whether they are significantly different from each other, at a simultaneous significance level of 5%. TUKEY's test (SCHEFFÉ 1959), which is more powerful, can only be applied if the number of observations are equal, in other cases Scheffé's test has been applied. It is to be remarked that with Scheffé's test no such pair of mean values could be distinguished, altough the analysis of variance gives a significant result The same test was applied to the data concerning the area of the nucleus, the cytoplasm, and the total area of the cell section

3. RESULTS

3.1. Morphological observations

3.1.1. The pollen mother cell stage until diplotene

The pollen mother cell has a large nucleus with a heterogeneous nucleolus. The plastids contain membranes. The mitochondria have few cristae and a spherical or rod-like shape The Golgi bodies produce only a few very small vesicles Vesicles with an electron transparent content are present (*fig. 1*). Some strands of rough endoplasmic reticulum (RER) are dispersed throughout the cyto-

plasm. Many ribosomes and polysomes are present The cell wall has plasmodesmata, during leptotene the cell wall is osmiophilic and thin.

During leptotene some lipid granules lie together and are connected with electron transparent vesicles and the whole group is surrounded by dark dots. This "lipid complex" is only visible after fixation with OsO_4 (*fig 2*)

During zygotene and pachytene the synaptinemal complex is present in the nucleus (*fig 3*) The nuclear pore is complex and has an annulus (*fig. 4*). In some places the presence of short extensions is suggested, as was described in *Pinus* (WILLEMSE 1971a) There are only few changes in the cell organelles: now the Golgi bodies produce more vesicles and mainly ribosomes are present. The callose wall formation starts Around the cell and against a thin layer of electron dense material the electron transparent callose wall appears The cellulose wall between the cells remains still visible The electron dense layer represents the thin new cell wall which is formed around each cell before meiosis starts In these cell walls channels are observed through which organelles or a part of the nucleus may pass (*fig 5*)

3.1.2 Diplotene until the tetrad stage

During diplotene the nucleolus begins to disappear, in the karyoplasm many granules appear. In the cytoplasm the volume of the plastids increases. Few lipid granules are now present The Golgi bodies produce very small vesicles and concentric membranes are also observed (*fig. 6, 7*). With $KMnO_4$ fixation the ER appears locally against the plasma membrane (*fig 7*) Microtubules are found near the plasma membrane mainly oriented in one direction The thin new cell wall can be observed around the thick callose wall in which also gaps are visible, representing the places where channels were present before (*fig. 6*).

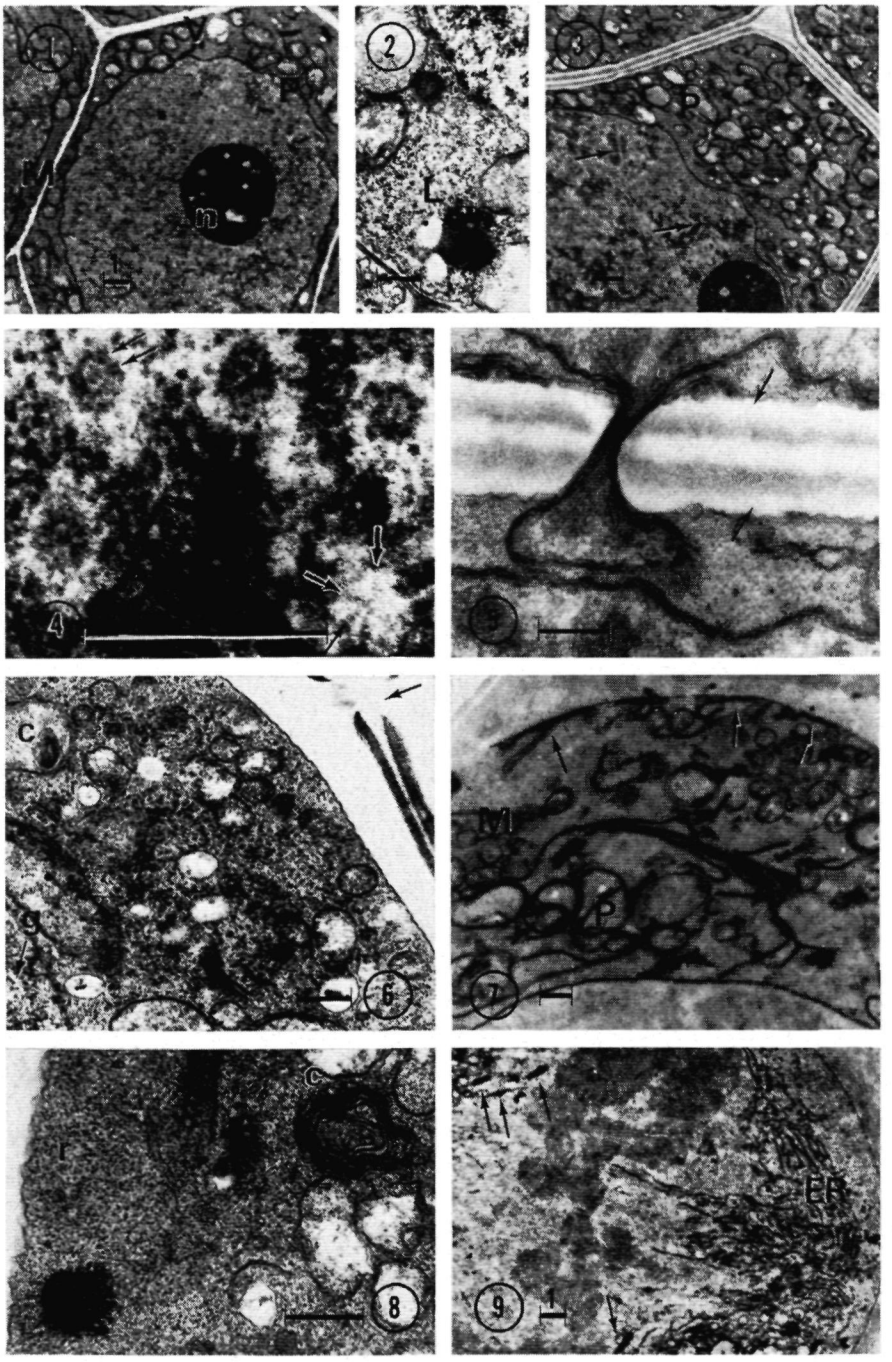
The tapetal cells show some orbicules outside the plasma membrane on which also electron dense granules are situated.

During the meiotic divisions the nuclear membrane is partly broken down. After the telophase the granules of the karyoplasm are mixed with the cytoplasm in which many ribosomes become visible Especially when the first meiotic division takes place many vesicles are present A centriole or polar body could not be observed. Remarkable is the presence of the ER strands in the polar region (*fig 9*) In the cytoplasm nucleolar-like bodies are frequently observed, concentric membranes (*fig 8*) remain present During the divisions the Golgi bodies are situated near the nucleus or nuclear region (*fig. 9, 10*). There may be a relation between the presence of Golgi bodies which produce small vesicles and the partly rebuilding of the nuclear membrane (*fig 11*).

During the short interphase II and prophase II the cell organelles lie between the two nuclei, whereas the mitochondria are mainly situated in the centre (*fig 10*)

3.1.3 The tetrad stage

After the separation of the four microspores by a callose wall the chromatine in the nucleus remains somewhat contracted for a while. Invaginations of the


1
n
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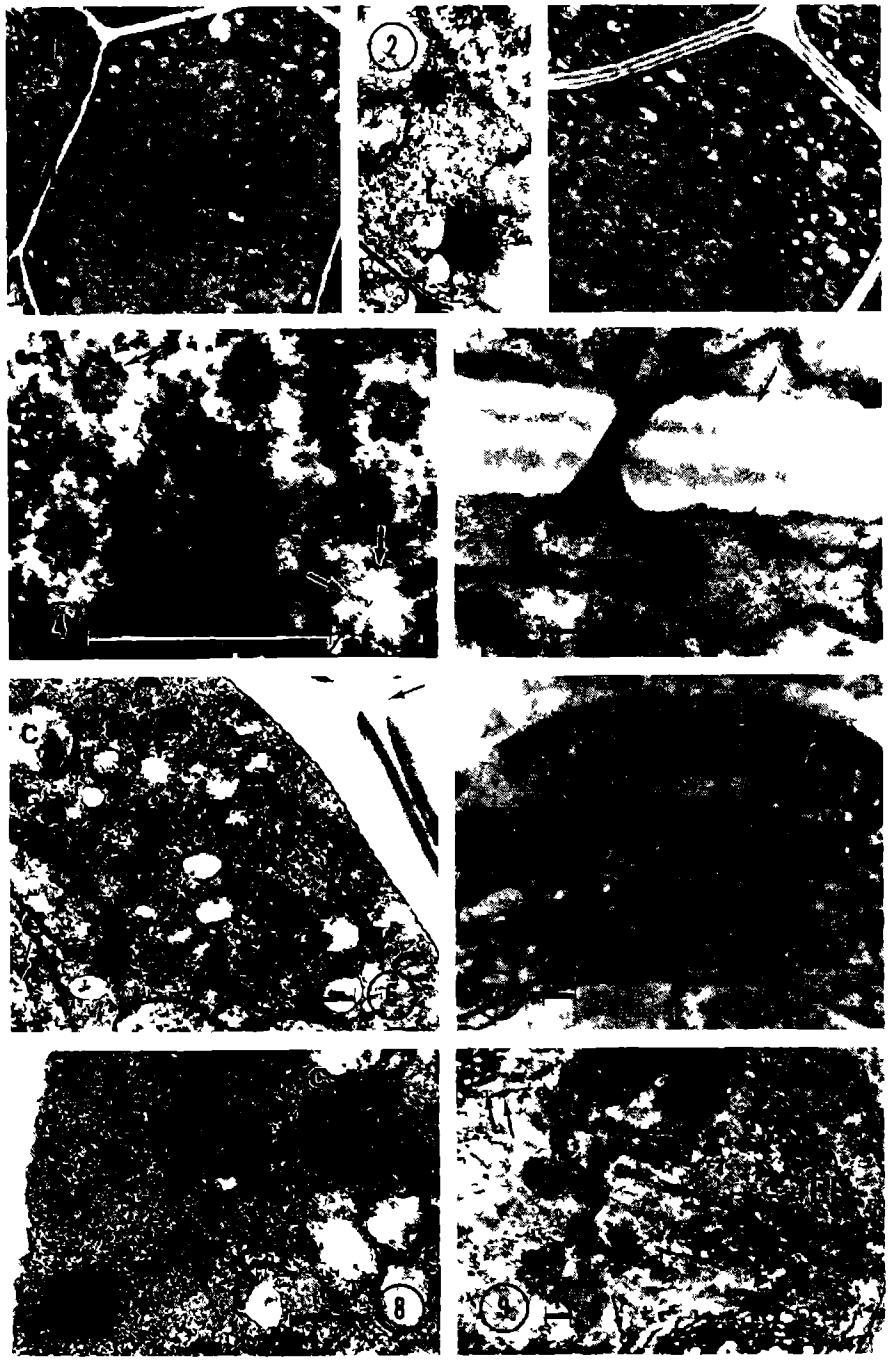
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nuclear membrane are not observed The nucleolar-like bodies remain visible in the cytoplasm (*fig 12*)

In some plastids electron transparent granules appear, probably as reserve food Many lipid granules are present when the pollen wall formation starts (*fig 12*) Mainly ribosomes are observed, some microtubuli and concentric membranes remain present The callose wall is still growing around the microspore until the excretion of the Golgi material takes place After poststaining electron dense spots become visible in the callose wall, probably on the border between the callose wall which was present before the tetrad stage and the one formed during the early tetrad stage (*fig 23*)

3 1 4 Pollen wall formation

During the early tetrad stage the plasma membrane lies against the straight callose wall (*fig 14*) In the cytoplasm the Golgi bodies start to produce electron transparent vesicles of different shape in which first an electron dense granule, thereafter fine fibrillar material is present (*fig 13*) The content of the Golgi vesicles is excreted and appears between the plasma membrane and the callose wall This excretion causes the undulations of the plasma membrane (*fig 15, 16*) The contact between the plasma membrane and the callose wall persists locally for a while In these places on the plasma membrane slightly electron dense material appears whereas the direct contact with the callose wall is severed In this way the bacula appear (*fig 17*) The excretion of the content of the Golgi vesicles continues and the plasma membrane retires further from the callose wall The places where the plasma membrane makes contact with the bacula extend and the bacula become cone-shaped The plasma membrane shows no undulations (*fig 18*) Electron dense material may be observed now on the place of the tectum The structure of bacula and tectum becomes well recognizable, their electron density seems to increase (*fig 19*) The contact on the base of the bacula with the plasma membrane becomes less intensive (*fig 20*) Now the footlayer is formed by the outgrowth of the base of the bacula The plasma membrane is no longer in contact with the bacula (*fig 21, 22*)

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Fig 1 Pollen mother cell with heterogeneous nucleolus (n), plastids (P), mitochondria (M) and vesicles (V), Glutaraldehyde (GA)-$KMnO_4$ fixation, × 3,680

Fig 2 Detail cytoplasm leptotene with "lipid complex" (L), ⩽ 13,000

Fig 3 Zygotene Note the synaptinemal complex (arrows), GA-$KMnO_4$ fixation, ⩽ 4,000

Fig 4 Nuclear pore with annulus and the short extensions (arrows), ⩽ 77,000

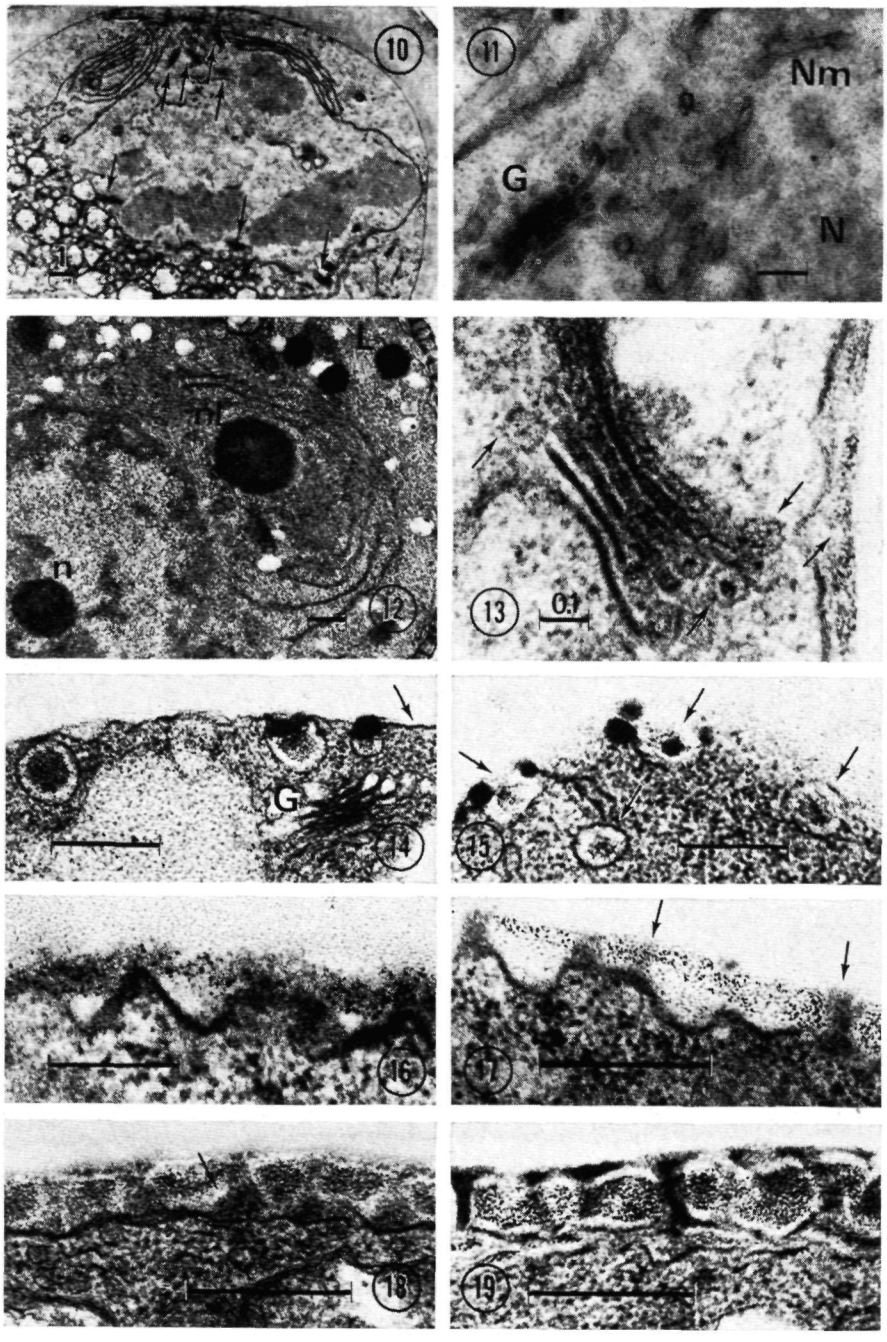
Fig 5 Zygotene cell wall channel with passing part of the nucleus Note the callose wall (arrow), GA-$KMnO_4$ fixation, ⩽ 22,600

Fig 6 Diplotene granules (g) in the karyoplasm Concentric membrane (c) and remnant of a channel between two cells (arrow), × 13,000

Fig 7 Diplotene ER along the plasma membrane (arrows), plastids (P) and mitochondria (M), GA-$KMnO_4$ fixation, × 8,300

Fig 8 Telophase II many ribosomes (r) and a concentric membrane (c), ⩽ 23,000

Fig 9 Early anaphase I in the polar region ER Golgi bodies around the nuclear region (arrows), GA-$KMnO_4$ fixation, ⩽ 3,600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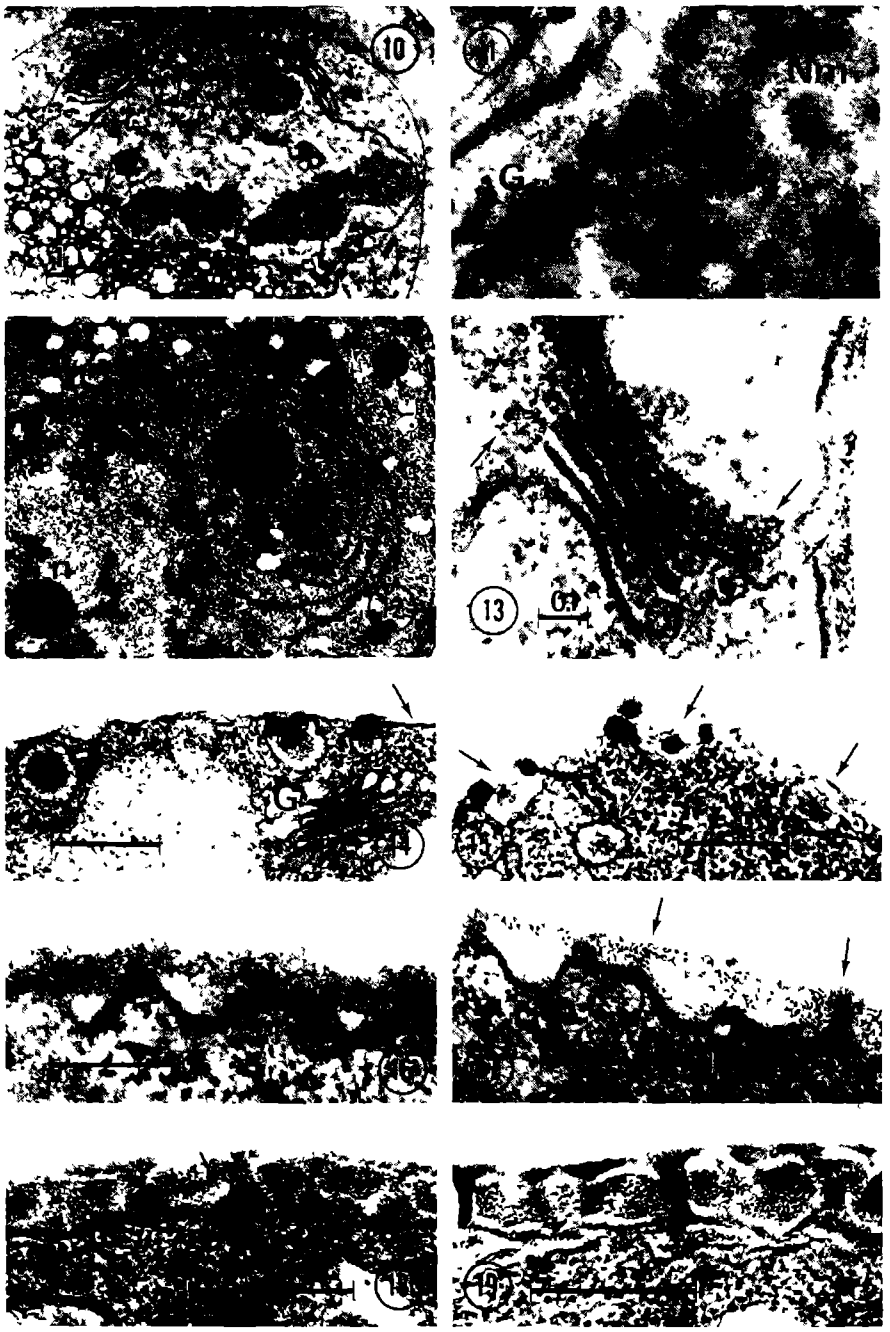
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Just before break-out of the microspore, the electron density in tectum, bacula and footlayer increases Between the plasma membrane and the footlayer and parallel to the plasma membrane thin membranes appear (*fig 23*) After break-out of the microspore tectum, bacula and footlayer are not yet thickening Probably the excretion of the Golgi material still takes place since Golgi vesicles are present in the cytoplasm Besides, material originating from Golgi vesicles lies between the plasma membrane and the footlayer (*fig 24*)

Around the young microspore the pollen wall thickens (*fig 25*) The material originating from the Golgi vesicles remains present between the bacula of the microspore wall Against the layer of Golgi material the pollen glue is visible on the pollen wall (*fig 26*) Lamellae of unit membrane dimension are only observed in the footlayer against the plasma membrane where the pollen wall sculpture is absent near the colpus area (*fig 27*)

In the cytoplasm the excretion of the content of the Golgi vesicles is blocked by a sheet of ER along the plasma membrane in the colpus area This sheet seems to be in contact with the nuclear membrane (*fig 28*)

During the tetrad stage orbicules and electron dense granules are observed along the plasma membrane outside the tapetal cell

3 1 5 The microspore before and after the first mitosis

The young microspore increases in volume and gets some vacuoles and vesicles in the cytoplasm All plastids contain electron transparent granules (*fig 29*) Golgi bodies produce many vesicles Packets of RER are formed in the cell principally near the nuclear membrane (*fig 30*) Ribosomes as well as polysomes are present The thin new cell wall remains intact after break-down of the callose wall and is visible against the tectum of the pollen wall for a long time Before mitosis the formation of the intine starts in the area of the colpus (*fig 29*) In this intine many thin extensions of the plasma membrane make contact with the

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Fig 10 Prometaphase II Golgi bodies around the nuclear region (arrows), GA-$KMnO_4$ fixation, × 2,900

Fig 11 Telophase II Golgi body (G) with vesicles near to the rebuilding nuclear membrane (Nm) N nucleus, GA-$KMnO_4$ fixation, × 13,000

Fig 12 Tetrad compare the nucleolar-like body (nl) with the nucleolus (n) L lipid granules, × 9,000

Fig 13 Golgi body during pollen wall formation Note the content of the Golgi vesicles and the material outside the plasma membrane (arrows), × 69,000

Fig 14 Start of pollen wall formation Golgi body (G) and plasma membrane against the callose wall (arrow), × 32,000

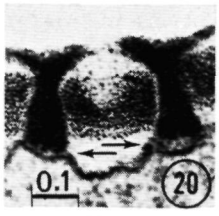
Fig 15 Excretion of the content of Golgi vesicles (arrows), × 33,000

Fig 16 Undulating plasma membrane, × 40,000

Fig 17 Appearance of the bacula (arrows), × 51,000

Fig 18 Cone-shaped baculum (arrow), no undulating plasma membrane Note the appearance of the tectum, × 47,000

Fig 19 Tectum and bacula get more contrast between the bacula material of the Golgi vesicles, × 47,000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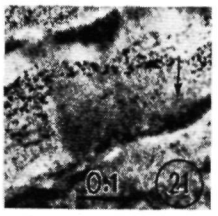
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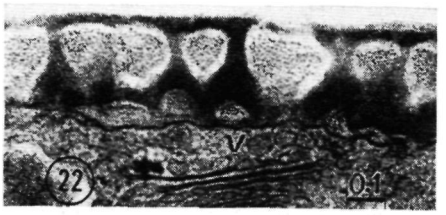
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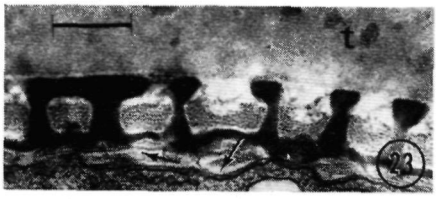
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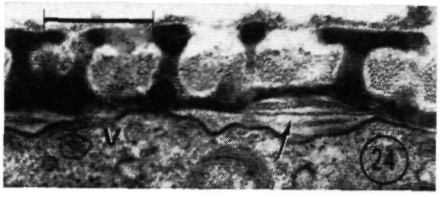
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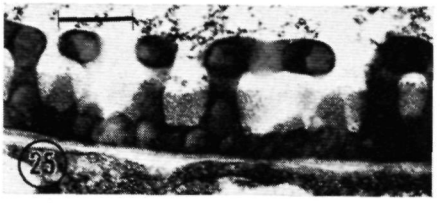
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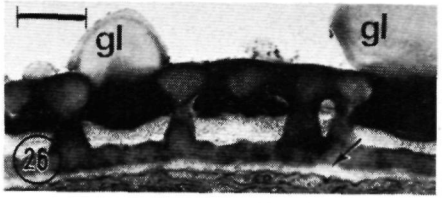
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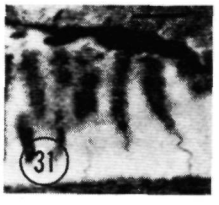
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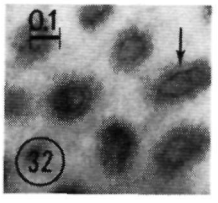
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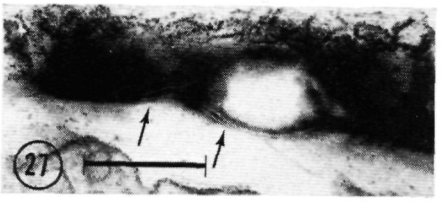
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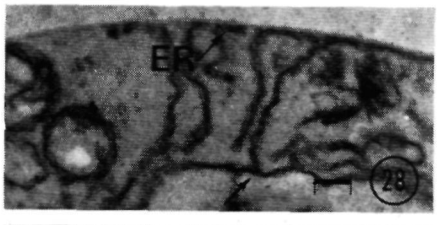
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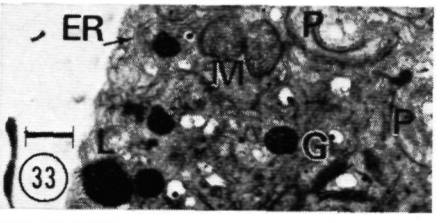
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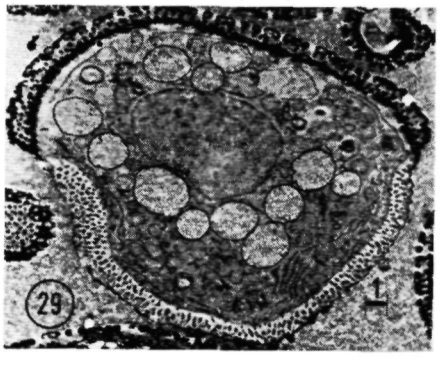
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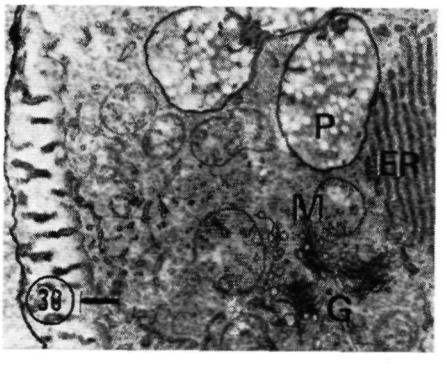
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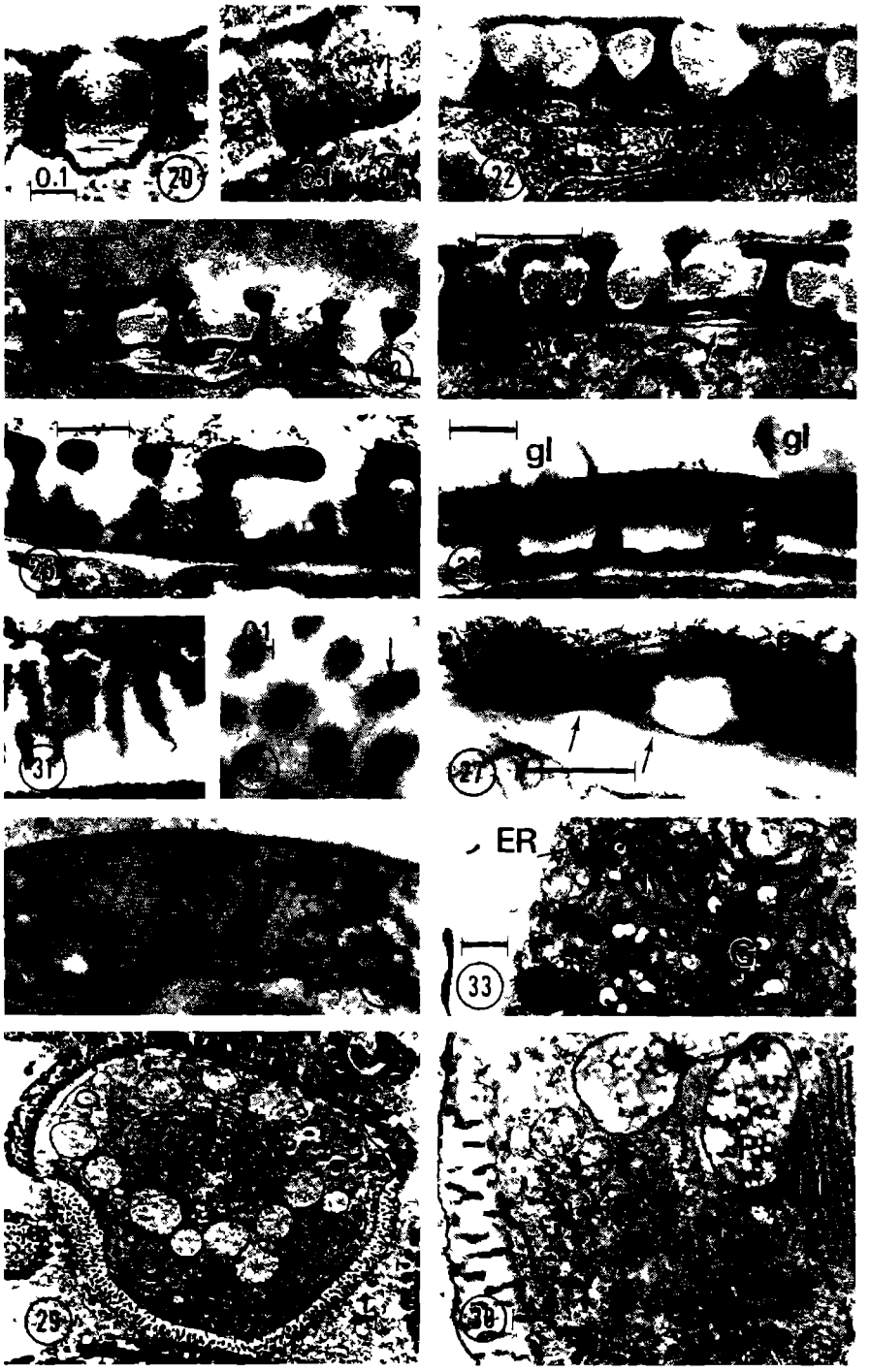
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surrounding fluid. The extensions are only covered by a thin layer of an unsculptured exine (*fig 31, 32*)

After mitosis, when the generative cell is formed, the cell organelles increase in number In the plastids the electron transparent granules begin to disappear The number of cristae in the mitochondria increases The Golgi bodies produce only a few small vesicles Many lipid granules appear Among the organelles are ribosomes and short strands of ER (*fig 33*) In the ripe pollen small vesicles become visible again (*fig 33*)

In the plastids of the tapetal cell electron dense globules are formed which come out into the fluid around the microspores during breakdown of the tapetal cell

3 2 Quantitative apoprach

Diagram 1 shows the changes in the cell section area, in the cytoplasm area, and in the nuclear area of the cell section during some different stages of microsporogenesis The mean values with standard deviations of the number of plastids, mitochondria, lipid granules, Golgi vesicles, and vesicles (or vacuoles) are noted per unit of area of the cytoplasm (100 points) In the diagram the results of the Tukey's test are also included

If the one way analysis of variance and the Tukey's test are applied to the number of cell organelles and to the cell section, cytoplasm, and nuclear area, significant results are obtained

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Fig 20 Contact between bacula and plasma membrane severs (arrows), × 57,000
Fig 21 Footlayer becomes visible (arrow), × 72,000
Fig 22 Formation of the footlayer by junction of the base of the bacula Note the Golgi vesicles (v), × 40,000
Fig 23 Before break-out of the microspore Membranes appear along the plasma membrane and the footlayer (arrows) Note the electron dense spots (t) in the callose wall, × 24,000
Fig 24 Pollen wall after break-out Note the membranes (arrow) and Golgi vesicles (v), × 33,000
Fig 25 Pollen wall of the young microspore, × 20,000
Fig 26 Pollen wall of the ripe microspore with intine (arrow) and pollen glue (gl), × 21,900.
Fig 27 Lamellae of unit membrane dimension (arrows), × 34,500
Fig 28 Early tetrad stage sheet of ER along the plasma membrane (arrow) Note the contact with the nuclear membrane (arrow), GA-$KMnO_4$ fixation, × 11,000
Fig 29 Young microspore Note the intine, GA-$KMnO_4$ fixation, × 2,800
Fig 30 Detail cytoplasm young microspore, plastids with electron transparent granules (P), mitochondria (M), Golgi bodies (G) and packet of ER, GA-$KMnO_4$ fixation, × 9,700
Fig 31 Extensions of the cytoplasm into the intine, GA-$KMnO_4$ fixation, × 12,500
Fig 32 Cross section of intine Note the plasma membrane (arrow), GA-$KMnO_4$ fixation, × 36,000
Fig 33 Cytoplasm of the ripe pollen Mitochondria with many cristae (M), plastids without electron transparent granules (P), lipid granules (L), Golgi bodies (G) and ER, × 14,800
Unless mentioned otherwise, the line on the figures represents a length of 0 5 μm, and OsO_4 fixation was used

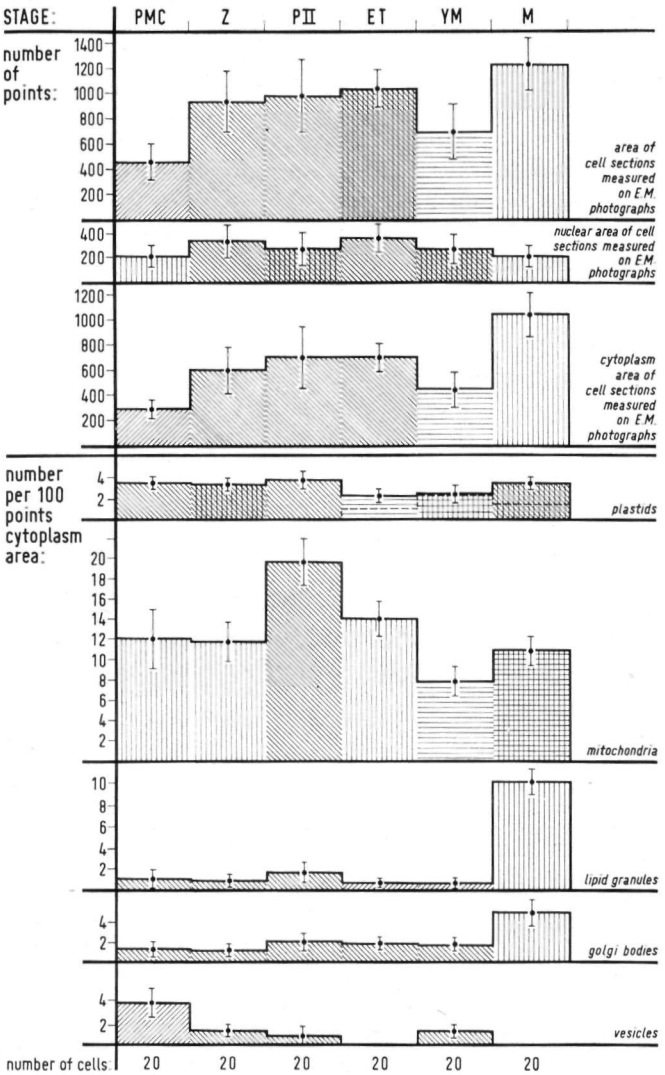

Diagram 1. The mean values and standard deviations of the cell section area, cytoplasm, and nuclear area of the cell section are given in number of points per stage of microsporogenesis as well as the number of organelles per 100 points cytoplasm area at the same stages. The dotted line represents the number of plastids in which electron transparent granules are present.

The results of the Tukey's test are indicated by means of an oriented shading. In columns with a corresponding type of shading the mean values do not differ significantly. A shading in two directions indicates that the mean value of this column does not differ significantly from all mean values of the columns with shading in each of the two directions of the double shaded column.

The number of cells counted is given on the base of the diagram.

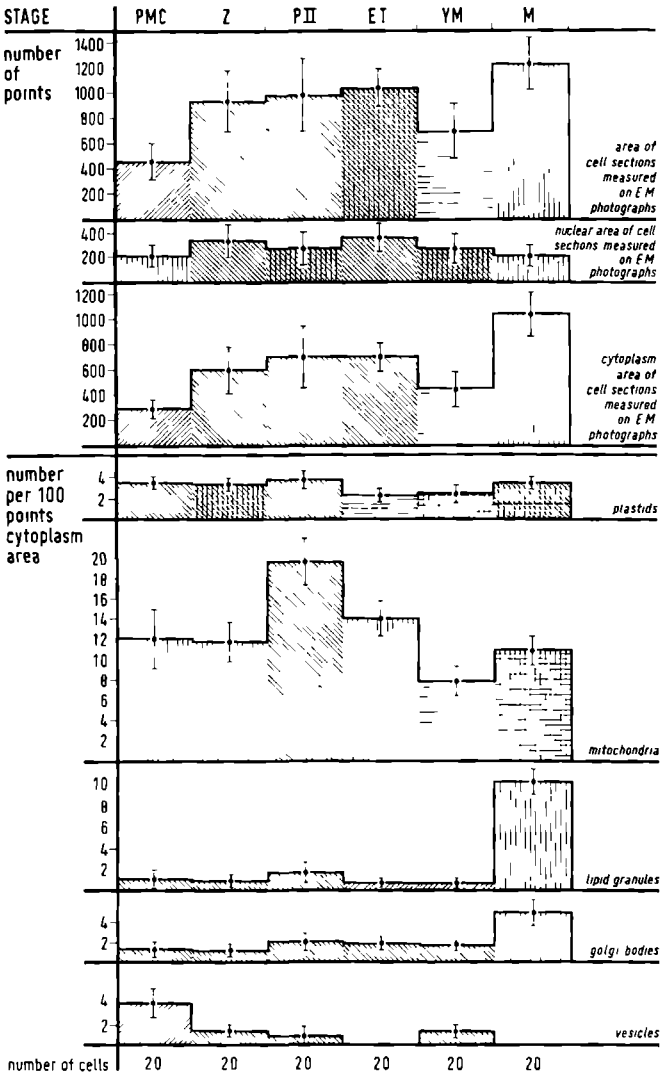

Diagram 1. The mean values and standard deviations of the cell section area, cytoplasm, and nuclear area of the cell section are given in number of points per stage of microsporogenesis as well as the number of organelles per 100 points cytoplasm area at the same stages. The dotted line represents the number of plastids in which electron transparent granules are present.

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The number of cells counted is given on the base of the diagram.

4 DISCUSSION AND CONCLUSION

The results obtained with *Gasteria* show differences as well as similarities with respect to the microsporogenesis described for other plants and in *Pinus sylvestris* in particular

4 1 Morphological changes in the nucleus

During zygotene and pachytene the synaptinemal complex is observed During diplotene an increase of granules in the karyoplasm starts which is followed by a mixing of this material with the cytoplasm during the telophase In telophase many ribosomes are present in the cytoplasm Both phenomena are also observed in other plants including *Pinus*

As in *Lilium* (DICKINSON & HESLOP-HARRISON 1970) in telophase I nucleolar bodies become visible in the cytoplasm In *Gasteria* the nucleolar-like bodies are found from telophase up to the young microspore stage In *Pinus* they are present from diplotene to interphase II In general the nucleolar-like bodies may function as carrier of nuclear information to the cytoplasm, this may occur partly instead of the formation of invaginations in the nuclear membrane as in *Podocarpus* (ALDRICH & VASIL 1970) or in *Pinus* (DICKINSON & BELL 1970, WILLEMSE 1971b)

4 2 Morphological changes in the cytoplasm

In plastids of *Gasteria* reserve material appears as electron transparent granules from the tetrad stage until after the first mitosis of the microspore The morphology as well as the time of appearance show no similarities with the presence of reserve material in the plastids of *Pinus* or *Tradescantia* (MARUYAMA 1968)

The cristae of the mitochondria increase in number after the first mitosis This has been observed also in *Pinus sylvestris* (WILLEMSE 1971e) and in other plants (HOEFERT 1969, DEXHEIMER 1970)

In *Gasteria* a "lipid complex" is only present in the leptotene and zygotene, no further aggregation of lipid granules occurs such as has been observed in *Lilium* (HESLOP-HARRISON & DICKINSON 1967) or *Pinus*

The Golgi bodies are always present Production of vesicles takes place during zygotene to form the callose wall, during the division stages to rebuild the nuclear membrane, during the tetrad stage to form the pollen wall, and during the young microspore stage to form the intine In *Pinus* the same relations and functions could be noted Whether one generation of Golgi bodies supplies all these functions or more generations of Golgi bodies exist, as has been suggested in *Tradescantia* (MARUYAMA 1968), could not be demonstrated in *Gasteria*

Vesicles appear in the pollen mother cell stage, zygotene, and after the breakout of the microspores During the cell divisions small vesicles are present in the cytoplasm

Packets of ER are observed only during the young microspore stage Strands of ER are found in every stage of development Concentric membranes are observed from diplotene to the young microspore stage, as may be partly the

case in *Pinus*, in *Gasteria* no relation could be established to the formation of new Golgi bodies as in *Tradescantia* (MARUYAMA 1968).

As in *Pinus*, ribosomes are always present in the cytoplasm of *Gasteria*. Polysomes are found in the pollen mother cell, during telophase with interphase II or early tetrad stage, and in the young microspore.

About the microtubules less information has been obtained.

As has been found in many other microspores, during the zygotene an intensive contact between the cells exists by means of channels (HESLOP-HARRISON 1971). In *Gasteria* the callose wall appears during the zygotene, in *Pinus* during the diplotene. The thin new cell wall around the microspore is also observed around the young microspores and seems to be resistant to the enzymes which break down the callose wall.

4.3. The pollen wall formation

In *Gasteria* the template of the pollen wall is formed by a local excretion of the content of Golgi vesicles and a brief contact between the plasma membrane and the callose wall. In the places where the contact between plasma membrane and callose wall persists, the production of material for the pollen wall starts immediately. First the bacula and thereafter the tectum and footlayer appear, still in contact with the plasma membrane. The area of the plasma membrane producing the material for the pollen wall increases during the pollen wall formation. As in *Pinus*, in *Gasteria* the template of the pollen wall pattern also depends on the local excretion of the content of Golgi vesicles and on the contact between the plasma membrane and the callose wall.

The material for the pollen wall originates probably from the cytoplasm of the microspore. In *Gasteria*, as in *Pinus*, many lipid granules are present during the pollen wall formation. The production of the material for the pollen wall takes place on the plasma membrane. Lamellae of unit membrane dimension are only observed in the basal layers of the footlayer near the intine of the colpus. Remarkable is the increase in electron density of the bacula and tectum during the formation of the pollen wall. The suggestion can be made that the pollen wall consists of two kinds of material: less electron dense material and electron dense material, which may be the sporopollenin. The other possibility is that the sporopollenin is preceded by a less electron dense precursor. The material for the pollen wall may be transported around the excreted Golgi material and may probably in this way reach the tectum and partly the footlayer.

Compared with the pollen wall formation in *Pinus sylvestris* the one in *Gasteria* shows some differences. In *Pinus* more of the material of the Golgi vesicles is excreted, however, for a shorter duration. This material is included in the pollen wall and precipitates against the protrusions of the callose wall which are formed on the plasma membrane. The callose wall formation in *Pinus* proceeds for a long time. Thereby the production of material for the pollen wall takes place on membranes partly inside and partly outside the cytoplasm. In *Gasteria* fewer Golgi vesicles are produced and the excreted material is probably only for a very small part included in the bacula, tectum, and foot-

layer The production of callose on the plasma membrane stops when the excretion of the content of the Golgi vesicles starts

From these results it appears that the pollen wall pattern and formation may depend on the quantity of excreted Golgi material, the duration of the callose wall formation, the start of the production of pollen wall material on the plasma membrane, and finally on the participation of the excreted Golgi vesicle material in the formation of the pollen wall

In *Pinus* the material of the Golgi vesicles is used both as material for the pollen wall and to push off the plasma membrane In *Gasteria* this material which remains in the pollen wall as a distinct layer between the bacula seems especially necessary for pushing off the plasma membrane and blocking further penetration of the pollen glue The material of the content of the Golgi vesicles probably consists of a polysaccharide and is less resistant than cellulose (WILLEMSE 1972) In both pollen types the excretion of the content of Golgi vesicles determines the pattern of the pollen wall

The participation of Golgi material in the formation of the pollen wall pattern may be deduced from the undulations of the plasma membrane, the presence of the content of Golgi vesicles outside the plasma membrane, and the presence of a sheet of ER in the area of the colpus which blocks the formation of a sculptured pollen wall because of the arrest of the Golgi vesicles Whether the transport of Golgi vesicles is active or passive is not clear

In *Pinus* and *Gasteria* the pollen wall thickens outside the callose wall during the young microspore stage During the pollen wall formation orbicules and electron dense granules are present outside on the plasma membrane of the tapetal cell The electron dense granules produced in the plastids of *Gasteria* may be a pigment or a pigment precursor for the anthocyanins (WIERMANN & WEINERT 1969)

4 4 The quantitative approach

Although a greater number of cells was counted per stage and fewer but more distinct stages could be used, all problems around the quantitative approach remain the same as discussed previously (WILLEMSE 1971c)

The area of the cell section and the cytoplasm area increase first from the pollen mother cell stage up to the tetrad stage and secondly from the young to the ripe microspore stage The area of the nucleus shows some fluctuations The area of the microspore is the largest During meiosis the area of the cytoplasm and of the total cell increases both in *Gasteria* and in *Pinus*

The density of cell organelles per unit of cytoplasm area of the pollen mother cell stage and the zygotene does not differ significantly except for the vesicles or the vacuoles This means that plastids, mitochondria, lipid granules and Golgi bodies increase about twice in number with the increase of the area of the cytoplasm A greater increase in number of the same organelles occurs in the microspore stage compared with the young microspore stage the density of organelles and the area of the cytoplasm increase Compared with the zygotene or the early tetrad stage the increase in number is less excessive for the plastids

and mitochondria The increase in number of cell organelles after the first mitosis has been reported in *Pinus sylvestris* (WILLEMSE 1971e) and in many other plants (SANGER & JACKSON 1971, VAZART 1971)

During the prophase II the density of cell organelles, except for the vesicles, is high This phenomenon is due to the localization of all organelles between the two nuclei The mitochondria are all located in the centre of the cell, whereas the plastids, vesicles and lipid granules are situated in the vicinity of the cell centre but between the two nuclei Golgi bodies lie around the nuclei Because of the presence of two nuclei in the selected cell sections a higher density of cell organelles per unit of cytoplasm may be expected Besides, no cell organelles disappear during the following stages

Except for the vesicles, no change in number of organelles occurs between the zygotene and the early tetrad stage The low number of plastids in the early tetrad stage is a border-line case (compare the mean values of the early tetrad stage and young microspore stage with the zygotene) This means that during meiosis no increase in the number of plastids, mitochondria, Golgi bodies, and lipid granules occurs The same has been demonstrated in *Pinus*

As could be expected, the decrease of the cytoplasm area during the young microspore stage and the constancy or decrease of the density of cell organelles per unit of cytoplasm reveal that the number of organelles has decreased in the young microspore cell, compared with the preceding stages

The density per unit of cytoplasm and number of vesicles or vacuoles during the microsporogenesis show great fluctuations

The average number of organelles per unit of cytoplasm area in *Gasteria* resp *Pinus* shows some similarities in the number of plastids and Golgi bodies However, in *Pinus* fewer mitochondria but more lipid granules are present than in *Gasteria*

Although in phylogenetical sense the pollen of *Gasteria* (Angiospermae, Monocotyledoneae) should be more highly developed than that of *Pinus* (Gymnospermae), the distinctness on a morphological level during microsporogenesis appears to be very small

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SAMENVATTING

Wanneer de uitvoerig beschreven resultaten van de morfologische veranderingen tijdens de microsporogenese van *Pinus sylvestris* vergeleken worden met die van *Gasteria verrucosa*, zijn er meer overeenkomsten dan verschillen aan te duiden Een belangrijke factor is het tijdsverschil waarmee de differentiatieprocessen verlopen, zoals onder andere de vorming van de callosewand, de vorming van de pollenwand, het verschijnen van de op de nucleolus gelijkende lichaampjes in het cytoplasma en van de groeperingen van vetdruppeltjes De volgende samenvatting betreft dan ook de microsporogenese van beide organismen

I VERANDERINGEN IN DE CELKERN VAN DE MICROSPORE

De kern ligt meestal centraal in de cel Tijdens de interfase II en profase II en het vroege tetrade stadium ligt de kern bij *Pinus* vlak tegen de celwand aan

In het vroege zygoteen zijn tussen het zich contraherende chromatine in *Pinus* op membranen gelijkende structuren zichtbaar, mogelijk ontstaan onder invloed van de ontmenging van het chromatine en karyoplasma, die dan aanvangt[1] In *Pinus* en *Gasteria* is het in structuur op elkaar gelijkend paringscomplex in veelvoud aanwezig tijdens het zygoteen en pachyteen

De nucleolus krijgt een heterogene structuur voor ieder delingsstadium In het diploteen begint de nucleolus te verdwijnen, terwijl er rondom granula zichtbaar worden Deze granula vullen geleidelijk het gehele karyoplasma op Na het verdwijnen van de kernmembraan worden deze granula met het cytoplasma gemengd, met name in de late telofase De granula worden beschouwd als voorlopers van ribosomen Juist tijdens en na de telofase komen er talrijke ribosomen in het cytoplasma voor Deze vernieuwing van de ribosoompopulatie heeft plaats na iedere kerndeling en is ook waargenomen in mitotisch delende cellen[1 2 6] Ook worden in het cytoplasma op de nucleolus gelijkende lichaampjes waargenomen, bij *Pinus* van diploteen tot de interfase II en bij *Gasteria* vanaf telofase I tot aan het jonge microspore stadium[2, 6]

Vóór de kerndelingen laat de kernmembraan golvingen zien en vervaagt ten dele Bij de opbouw van de kernmembraan tijdens de late telofase liggen rond de nog sterk gecontraheerde chromosomen zeer veel Golgi-lichaampjes die blaasjes produceren Delen van de oude kernmembraan worden mogelijk door het versmelten van deze Golgi-blaasjes opnieuw verbonden tot een aaneensluitende kernmembraan[2 6] In deze membraan liggen poriën met een annulus van acht, aan beide zijden regelmatig gerangschikte, uitsteeksels[2 6]

Tijdens de metafase worden de microtubuli aan het centromeer zichtbaar Een duidelijk polair centrum of centraallichaampjes zijn niet waargenomen, wel liggen er veel membraanstructuren in de polaire regio

In *Pinus* microsporen komen invaginaties van de kernmembraan voor in het vroege tetrade stadium Transport van materiaal uit de kern naar het cyto-

plasma via deze invaginaties is in verband gebracht met de vorming van de pollenwand[2].

2. De veranderingen in het cytoplasma van de microspore

Het cytoplasma met de organellen ligt tussen de kernen opgehoopt tijdens de late interfase II en profase II; in alle overige stadia ligt het rond de kern.

De plastiden bevatten in *Pinus*, uitgezonderd in het tetrade stadium, een zetmeelkorrel. Het verdwijnen van dit reservemateriaal tijdens het tetrade stadium is in verband gebracht met de vorming van de pollenwand. Tijdens het tetrade stadium tot aan de eerste mitose van de jonge microspore liggen er in de plastiden van *Gasteria* meerdere granula.

Bij beide organismen hebben de mitochondriën weinig cristae, deze nemen in aantal toe vlak voor de rijping van de microspore.

Vetdruppels zijn steeds in de microspore aanwezig. Tijdens de vorming van de pollenwand neemt het volume van deze druppels toe, mogelijk in verband met de productie van het sporopollenine[3, 6]. Groeperingen van vetdruppels samen met electronen doorlatende blaasjes treden in *Pinus* op van zygoteen tot diakinese en tijdens de tweede meiotische deling tot aan het midden van het tetrade stadium. In *Gasteria* is dit "complex van vetdruppeltjes" alleen in het leptoteen en zygoteen waargenomen. Mogelijk bestaat er een verband tussen de vorming van callose en het optreden van deze groeperingen van vetdruppeltjes[2, 6].

Het Golgi-lichaam vormt blaasjes en cisternen, deze transporteren en lokaliseren materiaal voor de callose- of de pollenwand. De vorming van een deels nieuwe kernmembraan is wellicht ook een gevolg van versmelting van Golgiblaasjes[2, 3, 6].

Grotere blaasjes met electronen doorlatende inhoud zijn talrijk tijdens de stadia waarin de kern zich deelt. Het volume van de blaasjes neemt toe vlak na het vrijkomen van de microsporen uit de tetrade; de blaasjes gaan dan over in kleine vacuolen.

Het endoplasmatisch reticulum, in *Pinus* van zygoteen tot diploteen in pakketjes gelegen, verliest vóór de eerste meiotische deling zijn ribosomen. Na de diakinese wordt in *Pinus* weinig endoplasmatisch reticulum waargenomen, dit in tegenstelling tot *Gasteria* waar steeds membranen zichtbaar blijven. In het vroege tetrade stadium houdt een schild van endoplasmatisch reticulum in de streek van de toekomstige colpe de uitscheiding van de inhoud van Golgiblaasjes tegen, waardoor hier de vorming van de sexine van de pollenwand wordt verhinderd. In *Gasteria* zijn pakketjes van endoplasmatisch reticulum bezet met ribosomen, alleen aanwezig in het cytoplasma van jonge microsporen tot vóór de eerste mitose[1, 6].

In alle stadia zijn ribosomen aanwezig in het cytoplasma van beide organismen. Na de telofase neemt het aantal ribosomen steeds sterk toe en treden er ook polysomen op[2]. Polysomen zijn aanwezig tot aan het diploteen, vanaf interfase II tot aan het midden van het tetrade stadium en in de jonge microspore.

Tussen de aanwezigheid van polysomen en de vorming van de callosewand bestaat mogelijk een relatie

Microtubuli verschijnen in het cytoplasma pas vanaf het zygoteen en liggen in alle richtingen Bij het verdwijnen van de kernmembraan komen de microtubuli ook voor in het karyoplasma en treedt er een gerichtheid op in verband met de vorming van de spoel

3 DE CELWANDEN VAN DE MICROSPORE

3 1 De nieuwgevormde celwand en de callosewand

Vlak voor het begin van de meiose is de pollenmoedercel omgeven door een dunne nieuwgevormde celwand van fibrillaire structuur Deze verschilt van de cellulose celwand, die in het zygoteen geleidelijk verdwijnt Na het vrijkomen van de microsporen uit de tetrade verdwijnt de nieuwgevormde celwand niet tegelijk met de callosewand, maar blijft nog lange tijd intact[1] [6]

De callosewand rondom de microspore wordt in *Pinus* aangelegd tijdens het diploteen, in *Gasteria* tijdens het zygoteen en pachyteen, waarbij tijdelijk in deze wand kanalen aanwezig zijn en de cellen het karakter van een syncytium krijgen In het vroege tetrade stadium wordt tussen de vier microsporen in centripetale richting een callosewand aangelegd[2] [6]

Golgi-lichaampjes produceren blaasjes en cisternen, die fijn fibrillair materiaal bevatten en dit transporteren buiten de plasmamembraan voor de vorming van een netwerk waarin later callose verschijnt Callose wordt gesynthetiseerd aan de plasmamembraan Dit blijkt uit de vorming van de lange uitsteeksels aan de callosewand bij de pollenwandvorming in *Pinus*[3], uit het geleidelijk dikker worden van de callosewand tot in het tetrade stadium, terwijl de uitscheiding van materiaal van de Golgi-blaasjes reeds gestopt is[2], en tenslotte uit gegevens van andere onderzoekers[2]

In het late tetrade stadium begint de afbraak van de callosewand De callosewand heeft een functie bij de vorming van de pollenwand en isoleert de zich ontwikkelende microspore van het omliggende diploide weefsel

3 2 De pollenwand

De vorming en de uiteindelijke structuur van de pollenwand van *Pinus*, met zijn windzakken en de tweelagige nexine, verschillen van die van *Gasteria* Bij beide organismen is het patroon van de pollenwand afhankelijk van de uitscheiding van materiaal uit de Golgi-blaasjes en van het lokaal contact tussen de callosewand en de plasmamembraan op plaatsen waar geen Golgi-materiaal wordt uitgescheiden Met het kortstondig blijvend contact tussen plasmamembraan en callosewand is de plaats van een baculum bepaald De uitscheiding van het granulaire, fijn fibrillaire Golgi-materiaal, een polysaccharide, blijkt uit de aanwezigheid van dit Golgi-materiaal langs de plasmamembraan, uit de golvingen van de plasmamembraan en uit het niet gevormd worden van de sexine wanneer de uitscheiding door een schild van endoplasmatisch reticulum of door een te smalle strook cytoplasma verhinderd wordt[3] [6]

Bij *Pinus* gaat tijdens het uitscheiden van het Golgi-materiaal de vorming van callose door op de plaatsen waar het contact tussen plasmamembraan en callosewand blijft bestaan Op deze wijze ontstaan uitsteeksels aan de binnenkant van de callosewand Tegen deze uitsteeksels en tegen de callosewand slaat het nu fibrillaire materiaal, afkomstig van de Golgi-blaasjes, neer Hierin verschijnt electronendicht materiaal, eerst op de plaats van het toekomstige tectum, vervolgens langs de uitsteeksels, de plaats van de bacula, en tenslotte langs de plasmamembraan, de footlayer Dit electronendicht materiaal, waarschijnlijk het sporopollenine, wordt geproduceerd aan membranen, hoofdzakelijk aan de plasmamembraan, maar ook aan membranen in het cytoplasma[3]

Bij *Gasteria* blijft het Golgi-materiaal buiten de plasmamembraan liggen en wordt niet opgenomen in de pollenwand De bacula verschijnen het eerst aan de sterk golvende plasmamembraan op de plaatsen waar het contact met de callosewand is blijven bestaan Vervolgens verschijnen de structuren van het tectum en de footlayer De electronendichtheid van deze wandstructuren neemt geleidelijk toe Ook in *Gasteria* heeft productie van electronendicht materiaal langs de plasmamembraan plaats

De sexine (tectum en bacula) en de nexine (footlayer) nemen in dikte toe wanneer de microsporen uit de tetrade vrijkomen Dit materiaal komt van buitenaf op de pollenwand terecht en is afkomstig van de microsporen en van de tapetumcellen Mogelijk is een voorstadium van de electronendichte stof, in de vorm van een electronen doorlatende stof, eerst aanwezig in de ruimte tussen de microsporen en tapetumcellen, voordat deze zich afzet op de pollenwand[3, 6]

Bij *Pinus* verschijnt er in de jonge microspore nog een laag onder de footlayer, de nexine II Bij *Gasteria* komt er op de volledig gevormde pollenwand nog een plakstof of "pollen glue" De windzakken bij *Pinus* pollen ontstaan door een lokaal meer uitscheiden van Golgi-materiaal en de vorming van lange callose-uitsteeksels aan de callosewand[3]

Ten opzichte van *Gasteria* wordt in *Pinus* meer en langere tijd Golgi-materiaal uitgescheiden en gaat de vorming van callose langer door Het electronendicht materiaal in *Pinus* verschijnt pas in het late tetrade stadium in grotere hoeveelheden Het verschil in de vorming van de pollenwand bij beide planten ligt voornamelijk in de wijze waarop de sexine wordt aangelegd

De intine wordt gevormd tijdens het jonge microspore stadium Bij *Gasteria* komen hierin kanalen voor[6]

4 DE TAPETUMCEL

De microsporen zijn omgeven door tapetumcellen, die een voedende functie hebben De tapetumcellen van *Pinus* en *Gasteria* behoren tot het secretorische type De tapetumcel van *Pinus* krijgt in het zygoteen geleidelijk veel ribosomen in het cytoplasma In het vroege tetrade stadium neemt dit aantal af, maar stijgt weer tijdens de vorming van de pollenwand rond de microsporen Door deze afwisseling zijn er twee cycli te onderscheiden in de ontwikkeling van de tapetumcel· van zygoteen tot het vroege tetrade stadium en van het midden tetrade stadium tot de degeneratie van de tapetumcel

De kern krijgt veel granula in het karyoplasma in het zygoteen en vroege tetrade stadium, mogelijk in verband met de toename van het aantal ribosomen in het cytoplasma. In het cytoplasma zijn op de nucleolus gelijkende lichaampjes waargenomen.

Plastiden hebben zelden zetmeelkorrels. De mitochondriën hebben ten opzichte van die in de microsporen meer cristae. Vetdruppeltjes zijn nauwelijks aanwezig. Productie van Golgi-blaasjes met electronen doorlatende inhoud heeft voortdurend plaats, de functie hiervan is niet duidelijk. Er treedt vacuolisatie in de tapetumcel op vóór het uiteenvallen van de cel en het vrijkomen van de celorganellen tussen de jonge microsporen[4].

Langs de plasmamembraan komen electronendichte globuli en "orbicules" voor. De vorming van de "orbicules" heeft plaats in het cytoplasma en staat in verband met het endoplasmatisch reticulum en polysomen, die het "pro-orbicule" omgeven. Buiten de plasmamembraan vormt zich om de "orbicules" een electronendichte laag, het sporopollenine. De globuli langs de plasmamembraan, de "orbicules" en de vorming van de zogenaamde pollenzak, zijn aanduidingen van het vermogen van de tapetumcel om sporopollenine te produceren[4].

5. DE ZELFFLUORESCENTIE VAN DE POLLENWAND

De pollenwand alsook de electronendichte globuli en de "orbicules" van de tapetumcel laten een overeenkomende zelffluorescentie zien wanneer deze elementen worden bestraald met ultraviolet licht[5].

Het blijkt dat voornamelijk de sexine en de footlayer van de pollenwand de zelffluorescentie vertonen. Tijdens de vorming van de pollenwand treden er veranderingen op in het spectraal maximum, de intensiteit en uitdoving van de zelffluorescentie. De zelffluorescentie en de verandering tijdens de vorming van de pollenwand blijken voor elk type pollenkorrel specifiek te zijn. De intensiteit van de zelffluorescentie neemt in het algemeen toe na het vrijkomen van de microsporen uit de tetrade.

De zelffluorescentie van de pollenwand verandert onder invloed van diverse chemische behandelingen. Het blijkt dat het spectraal maximum van de zelffluorescentie verschuift en de intensiteit toeneemt wanneer de intine is verwijderd. Als onder invloed van sterke oxydatie in een zuur milieu het sporopollenine in de pollenwand van *Pinus* verdwijnt, blijkt ook de electronendichtheid van de pollenwand èn de zelffluorescentie te verdwijnen, terwijl de vorm van de pollenwand langer intact blijft. Het sporopollenine komt dus in de pollenwand naar voren als een electronendichte stof en heeft zelffluorescentie. Ten opzichte van *Gasteria* blijkt de pollenwand van *Pinus* minder bestand tegen dezelfde chemische behandelingen[5].

Het blijkt uit de vorming van de pollenwand en uit de verandering in zelffluorescentie dat de pollenwand zowel chemisch als structureel uit meerdere componenten is samengesteld. Bij *Pinus* bestaan de sexine en nexine I hoofdzakelijk uit Golgi-materiaal, een polysaccharide, dat later geïmpregneerd wordt

met sporopollenine; de nexine II bestaat uit een andere, onbekende electronendichte substantie. Bij *Gasteria* bevindt zich tussen de bacula het Golgimateriaal, een polysaccharide; de sexine bestaat eerst uit minder electronendicht materiaal, waarin later tegelijk met de vorming van de nexine het electronendichte sporopollenine verschijnt.

6. KWANTITATIEVE BENADERING VAN DE VERANDERING IN CELVOLUME EN CELORGANELLEN

Van zygoteen tot aan het jonge microspore stadium blijkt bij *Pinus* de oppervlakteverandering van de grootste doorsnee van intacte microsporen, gemeten met het lichtmicroscoop, een positieve correlatie te hebben met de verandering van het totale celoppervlak van een celdoorsnee van een microspore, gemeten met het electronenmicroscoop. Bij *Pinus* en *Gasteria* neemt vanaf het zygoteen tot na de eerste mitotische deling het volume van de cel toe. Dit is een toename van het volume van de kern èn van het cytoplasma[3, 6].

Vanaf zygoteen tot aan het einde van het tetrade stadium verandert het aantal plastiden, mitochondriën, vetdruppels en Golgi-lichaampjes in het cytoplasma niet. Een vermeerdering in het aantal van deze organellen heeft in *Gasteria* plaats vóór het leptoteen en na de eerste mitose van de jonge microspore, tegelijk met de volumetoename van de cel[6]. Het blijkt dat de jonge microspore voor een groot deel cytoplasma bezit van diploide oorsprong. De haploide kern functioneert aanvankelijk in een merendeels diploid cytoplasma.

Per eenheid van cytoplasma-oppervlak heeft *Pinus* gemiddeld minder mitochondriën, maar meer vetdruppeltjes dan *Gasteria*. De aantallen plastiden en Golgi-lichaampjes komen overeen. De dichtheid van deze organellen per eenheid van cytoplasma-oppervlak neemt pas na de eerste mitose toe[3, 6].

Alhoewel bij *Pinus* en *Gasteria* verschillende reeksen van stadia in een verschillend aantal cellen zijn geteld, komen de kwantitatieve resultaten overeen.

Van Professor Dr J M Denuce, Hoofd van het Zoologisch Laboratorium van de Katholieke Universiteit te Nijmegen, heb ik, als medewerker van dit Laboratorium, de volledige vrijheid gekregen om deze dissertatie te kunnen voltooien

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STELLINGEN

I

Het patroon van de pollenwand wordt hoofdzakelijk bepaald door uitscheiding van materiaal uit Golgiblaasjes en het contact tussen de plasmamembraan en de callosewand.

Dit proefschrift.

II

De zelffluorescentie van de sexine van de pollenwand verandert tijdens de microsporogenese en is voor ieder type pollenkorrel specifiek.

Dit proefschrift.
M. TH. M. WILLEMSE (1971): In: Sporopollenin, Academic Press, London.

III

Het verwerven en adequaat beheren van uitgestrekte, voor flora en fauna van een gewest representatieve, natuurreservaten is een weliswaar noodzakelijk maar niet toereikend middel voor verwezenlijking van de doeleinden van het natuurbehoud.

IV

De plaats waar ectodermaal weefsel tijdens de embryonale ontwikkeling invagineert, dient men te beschrijven als blastoporus. Aangezien bij enige beenvissen de invaginatie niet optreedt, ontbreekt de blastoporus.

W. W. BALLARD (1969): Pubbl. Staz. Zool. Napoli 37: 1–17.

V

Voor het bewijs van zijn DNA replicatiemodel dient WERNER meer rekening te houden met de transdeoxyribosidase werking.

R. WERNER (1971): Nature 230: 570–572.

VI

De inductie van de temperatuurpuffs bij Drosophila hydei heeft waarschijnlijk een mitochondriale oorsprong.

H. J. LEENDERS (1971): Dros. Inf. Service 46: 64.

VII

De invoering van het begrip progymnospermen als schakel in een fylogenetische afstamming van de zaadplanten is niet verantwoord en in tegenspraak met de afstamming van de varens wanneer met het begrip de onderklasse van de Primofilices wordt bedoeld

F Ehrendorfer (1971) In Lehrbuch der Botanik fur Hochschulen, Gustav Fischer Verlag, Stuttgart, 30e druk p 599

VIII

Bij oecologisch onderzoek dient aandacht besteed te worden aan het verband tussen dagritme en migratie van amfibieën

W Himstedt (1971) Oecologia (Berl) 8 194–208

IX

Bij het binnendringen van de pollenbuis van Pinus sylvestris in het nucellusweefsel worden de celwanden door enzymen, die voor de pollenbuis uitgaan, aangetast

M Th M Willemse & H F Linskens (1969) Rev Cytol Biol veg 32 121–128

X

Bij de nomenclatuur van de stratificatie van de pollenwand van fossiele pollenkorrels dient men rekening te houden met de aantasting van de pollenwand

XI

De meeste reclames betreffende de zogeheten zachte contactlenzen moeten gekwalificeerd worden als onjuiste voorlichting

XII

De aandacht, besteed aan de aankomst van keizer Wilhelm II te Eysden op 10 november 1918, staat in geen verhouding tot de historische importantie ervan

XIII

In de zin: 'Voor het eerst in zijn geschiedenis is de mens in staat zijn eigen evolutie doelbewust te richten', wordt de mens niet alleen buiten maar zelfs boven de evolutie geplaatst.

A. J. H. THIADENS (1969): De stamboekmens die niet sterven mag. Paul Brand, Hilversum, p. 1.

XIV

In verband met rellen in Amsterdam verdient het aanbeveling in de Nederlandse taal omtrent de afkorting S.J. een duidelijke afspraak te maken.

XV

Te vroege en te ver gaande specialisatie leidt tot isolatie en communicatiestoornissen in de wetenschap.

XVI

Stellingen zijn niet geschikt voor publicatie in een dagblad.

M. TH. M. WILLEMSE
24 maart 1972